

# TRAITE DE COOPERATION EN MATIERE DE BREVETS

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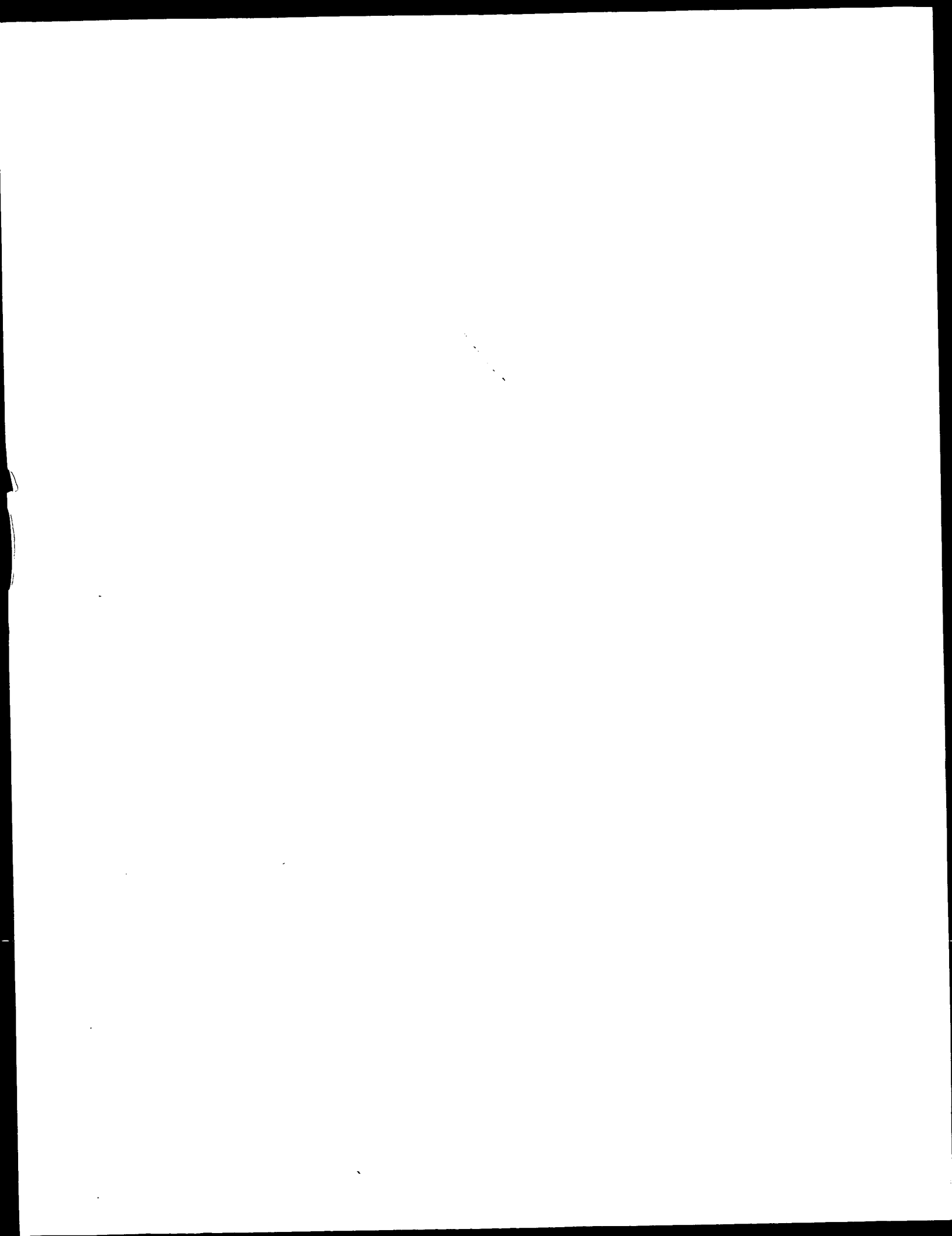
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(54) Title: USE OF ULIP PROTEINS IN THE DIAGNOSIS AND THERAPY OF CANCER AND PARANEOPLASTIC NEUROLOGICAL SYNDROMES

(54) Titre: UTILISATION DES PROTEINES ULIP DANS LE DIAGNOSTIC ET LA THERAPIE DES CANCERS ET DES SYNDROMES NEUROLOGIQUES PARANEOPLASIQUES

## (57) Abstract

The invention concerns a purified polypeptide, biologically active polypeptide derivative or fragment of said purified polypeptide, comprising an amino acid sequence selected among SEQ.ID. No. 2, No. 4, No. 6 and No. 8.

## (57) Abrégé

L'invention a pour objet un polypeptide purifié, dérivé ou fragment polypeptidique dudit polypeptide purifié biologiquement actif, comprenant une séquence d'acides aminés choisie parmi SEQ.ID. n°. 2, n°. 4, n°. 6 et n°. 8.

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Utilisation des protéines ULIP dans le diagnostic et la thérapie des cancers et des syndromes neurologiques paranéoplasiques".

L'invention concerne l'utilisation des protéines dénommées ULIP/POP dans le diagnostic et la thérapie des cancers et des syndromes neurologiques paranéoplasiques.

Les syndromes neurologiques paranéoplasiques (SNP) surviennent à l'occasion d'un cancer, souvent avant sa découverte et ne sont liés ni à la prolifération tumorale elle-même (envahissement direct, métastases) ni à la thérapie. Leur fréquence est globalement estimée à environ 1 % des cancers. Plusieurs tableaux cliniques ont été depuis longtemps individualisés (encéphalomyélite, neuropathie sensitive de Denny Brown, atrophie cérébelleuse, encéphalite limbique, opsoclonus, ...) correspondant en fait à l'atteinte soit élective soit préférentielle de certains groupes de neurones. La fréquence des cellules inflammatoires au voisinage des lésions avait fait évoquer depuis de nombreuses années la possibilité d'un processus auto-immun ou viral. La mise en évidence, plus récente, d'auto-anticorps dans le sérum et le liquide céphalo-rachidien (LCR) de patients souffrant de SNP, spécifiques du type de tumeur et du type de neurones qui dégénèrent, a relancé l'hypothèse d'une participation de l'auto-immunité dans la genèse de cette pathologie (Graus et al., 1985 ; Greenlee et al., 1983).

Outre la présence d'un titre élevé de ces anticorps dans le sang et le LCR des patients, il existe plusieurs arguments suggérant que les SNP relèvent de mécanismes auto-immuns. Ainsi, les antigènes reconnus dans le système nerveux central sont aussi présents dans les tumeurs des patients (Anderson et al., 1987). Au sein du tissu tumoral, on retrouve des anticorps spécifiquement dirigés contre ces antigènes ainsi que des lymphocytes B et T (Hetzl et al., 1990).

Ces données suggèrent que le processus auto-immun serait déclenché par l'expression d'antigènes tumoraux. Un processus d'immunité croisée provoquerait les lésions du système nerveux central. D'autres arguments indiquent en outre que les lésions cérébrales résultent de la réponse auto-immune. Ainsi, dans le cerveau des patients, le titre des anticorps spécifiques est supérieur à celui du sérum et du LCR (Dalmau et al., 1991). De plus, dans le cas des encéphalomyélites associées aux anticorps anti-Hu, il existe une réaction lymphocytaire intense, composée de cellules B et T, située à proximité de neurones en voie de destruction (Dalmau et al., 1991 ; Graus et al., 1990).

Plusieurs types d'auto-anticorps permettant des regroupements syndromiques précis en fonction de critères immunologiques, neurologiques et carcinologiques ont été décrits.

Ainsi, les anticorps anti-Yo sont retrouvés dans le sérum et le LCR de femmes présentant une atrophie cérébelleuse paranéoplasique et un cancer gynécologique (ovaire, sein ou utérus) (Greenlee et al., 1983 ; Jaeckle et al., 1985).

Ces anticorps reconnaissent deux protéines cytoplasmiques de 34 et 62 kDa spécifiques des cellules de Purkinje du cervelet.

Les anticorps anti-Ri sont retrouvés dans le sérum et le LCR de patients (principalement des femmes) présentant un opso-myoclonus, un syndrome cérébelleux et un cancer du sein. Ces anticorps reconnaissent deux protéines de 50 et 80 kDa spécifiques des neurones du système nerveux central (Luque et al., 1991).

Les anticorps anti-Hu sont les plus fréquemment rencontrés au cours des SNP. Ils sont retrouvés dans le sérum et le LCR de patients présentant un syndrome de Denny-Brown ou une encéphalomyélonévrite

et un cancer du poumon à petites cellules (Graus et al., 1985 ; Dalmau et al., 1992). Ces auto-anticorps reconnaissent plusieurs protéines de 37 à 45 kDa exprimées spécifiquement par l'ensemble des neurones du système nerveux.

5                   Récemment a été identifié chez des patients présentant un SNP un autre type d'auto-anticorps : les anticorps anti-CV2 (Antoine et al., 1993 ; Honnorat et al., 1996). Ces derniers sont atypiques, en ce sens que la cible antigénique reconnue à l'âge adulte est essentiellement non neuronale, alors que l'analyse du cerveau *post-mortem* de quatre patients  
10 permet d'objectiver une perte neuronale, une gliose et un processus inflammatoire caractéristique des SNP.

L'originalité de la découverte de ces auto-anticorps réside, d'une part, dans leur mise en évidence. Ces derniers avaient échappé à l'ensemble des investigations habituelles qui consistaient à  
15 révéler les antigènes reconnus par immunohistochimie sur du cerveau *post-mortem*. L'antigène reconnu est en effet soluble et disparaît du cerveau *post-mortem* dans la plupart des conditions de fixation. Seule une fixation du tissu *post-mortem* humain par immersion dans le paraformaldéhyde ou *in situ* par perfusion de paraformaldéhyde chez  
20 l'animal, a permis de révéler la présence de ces anticorps dans le LCR ou le sérum des patients atteints de SNP (Antoine et al., 1993 ; Honnorat et al., 1996).

Les auto-anticorps anti-CV2 présents dans les sérums de patients atteints de syndrome neurologique paranéoplasique (SNP) ont  
25 été définis par leur capacité à reconnaître, par immunohistochimie indirecte, un antigène cytoplasmique exprimé spécifiquement, dans le

cerveau de rat adulte, par une sous-population d'oligodendrocytes du tronc cérébral, de la moelle et du cervelet.

L'originalité de ces auto-anticorps réside, d'autre part, dans leur intérêt diagnostique. Leur présence dans le sérum ou le LCR de patients a valeur diagnostique puisqu'elle permet de préciser l'origine paranéoplasique d'un syndrome neurologique. La découverte de ces anticorps lorsqu'elle précède celle du cancer, oriente la recherche de celui-ci et permet sa découverte. Tel a été le cas pour six patients sur 19 présentant des anticorps anti-CV2. Les troubles cliniques étaient différents suivant les patients, certains présentaient un tableau d'encéphalite limbique, d'autres une encéphalomyélonévrite et d'autres un syndrome de Lambert-Eaton. Néanmoins, dans plus de 60 % des cas, le syndrome cérébelleux était prédominant. La tumeur la plus fréquemment associée était le cancer du poumon à petites cellules (60 % des cas).

Des expériences sur des cerveaux de rats nouveaux-nés ont montré que ces anticorps anti-CV2 réagissaient avec une protéine de 66 kDa (Honnorat et al., 1996).

Cet antigène se situe dans le cerveau adulte dans une sous-population d'oligodendrocytes ou dans des cellules qui gardent des capacités de différenciation dans le cerveau adulte (bulbe olfactif, gyrus denté). L'antigène reconnu jouerait un rôle dans la survie neuronale, via des interactions Neurone/Oligodendrocyte, comme le suggère la perte des neurones observée dans le cerveau *post-mortem* de patients atteints de SNP.

Son expression très restreinte à l'âge adulte contraste avec une expression très forte et transitoire dans le système nerveux central et

périphérique en développement, suggérant le rôle probable de cet antigène dans le développement du système nerveux.

La Demanderesse a caractérisé l'antigène cible des auto-anticorps anti-CV2 qui correspond à une protéine ci-après désignée par  
5 «POP-66» pour « paraneoplastic oligodendrocyte protein 66 kDa ».

De manière surprenante, il a été découvert que la protéine POP-66 appartient à la famille des protéines dites ULIP (pour Unc-33 like phosphoprotein), impliquée dans le contrôle du développement neuronal et le transport axonal, (T. Byk et al., 1996) et étudiée aussi sous la forme  
10 des protéines CRMP (Goshima et al., 1995, Wang et al., 1996), TOAD-64 (Minturn et al., 1995) et DRPs (Hamajima et al., 1996). Plus précisément, POP-66 a été identifiée comme étant en fait la forme humaine de ULIP-4.

L'ensemble des données décrit ci-après souligne la complexité de cette famille de protéines, l'existence d'un spectre  
15 d'expression très large des membres de cette famille dans le cerveau au cours de l'ontogénèse, mais très restreint chez l'adulte, ainsi que la spécificité des anticorps anti-CV2 pour un membre de cette famille protéique ULIP, qui est en fait POP-66.

Ainsi, la Demanderesse a montré que la protéine reconnue  
20 par les anticorps anti-CV2 de patients atteints de SNP est POP-66/ULIP-4 et a établi l'implication des protéines ULIP dans les syndromes neurologiques paranéoplasiques et les cancers associés. Outre leur rôle dans les cancers associés aux SNP, la Demanderesse a également découvert que les protéines de la famille ULIP pourraient jouer un rôle  
25 dans toute autre forme de cancer, non associée aux SNP. Plus particulièrement, les protéines ULIP seraient notamment impliquées dans

les cancers de tissus ayant une origine embryonnaire commune avec le système nerveux central.

La présente invention a donc pour objet un polypeptide purifié ULIP, dérivé ou fragment polypeptidique dudit polypeptide purifié.  
5 comprenant une séquence d'acides aminés choisie parmi SEQ ID n° 2, n° 4, n° 6 et n° 8.

De manière préférentielle, la présente invention a pour objet un polypeptide purifié, dérivé, ou fragment polypeptidique biologiquement actif dudit polypeptide purifié, comprenant la séquence d'acides aminés  
10 SEQ ID n° 8, ledit polypeptide étant désigné par « POP-66/ULIP-4 ».

Un fragment du polypeptide de séquence SEQ ID n° 8 d'intérêt est en particulier le fragment antigénique PARASCPGKIS (acides aminés n° 517 à n° 527).

Dans la description de l'invention, on utilise les définitions  
15 suivantes :

- dérivé : tout polypeptide variant du polypeptide de séquence SEQ ID n° 2, n° 4, n° 6 ou n° 8 ou toute autre molécule résultant d'une modification de nature génétique et/ou chimique de la séquence SEQ ID n° 2, n° 4, n° 6 ou n° 8, c'est-à-dire obtenue par  
20 mutation, délétion, addition, substitution et/ou modification chimique d'un seul ou d'un nombre limité d'acides aminés, ainsi que toute séquence isoforme, c'est-à-dire une séquence identique à la séquence SEQ ID n° 2, n° 4, n° 6 ou n° 8 à l'un de ses fragments ou séquences modifiées, contenant un ou plusieurs acides aminés sous la forme d'énantiomère D.  
25 lesdites séquences variantes modifiées ou isoformes ayant conservé au moins l'une des propriétés les rendant biologiquement actives.

- Biologiquement actif : présentant des propriétés d'induction et/ou de contrôle du développement neuronal et/ou des propriétés antigéniques.

L'invention a également pour objet une séquence d'acides nucléiques isolée choisie parmi SEQ ID n° 1, n° 3, n° 5 et n° 7 ou une  
5 séquence dérivée des séquences SEQ ID n° 1, n° 3, n° 5 et n° 7 du fait de la dégénérescence du code génétique, ou du fait de mutation, de délétion ou d'insertion d'au moins un nucléotide, lesdites séquences dérivées ayant une activité biologique pratiquement identique à celle du  
10 peptide codé par les séquences SEQ ID n° 1, n° 3, n° 5 et n° 7.

Les différentes séquences nucléotidiques de l'invention peuvent être d'origine artificielle ou non. Il peut s'agir de séquences d'ADN ou d'ARN, obtenues par criblage de banques de séquences au moyen de sondes élaborées sur la base des séquences choisies parmi SEQ ID n° 2,  
15 n° 4, n° 6 et n° 8. De telles banques peuvent être préparées par des techniques classiques de biologie moléculaire, connues de l'homme de l'art.

Les séquences nucléotidiques selon l'invention peuvent également être préparées par synthèse chimique, ou encore par des méthodes mixtes incluant la modification chimique ou enzymatique de  
20 séquences obtenues par criblage des banques.

Ces séquences nucléotidiques permettent la réalisation de sondes nucléotidiques, capables de s'hybrider fortement et spécifiquement avec une séquence d'acides nucléiques, d'un ADN génomique ou d'un ARN  
25 messenger, codant pour un peptide selon l'invention ou un fragment biologiquement actif de celui-ci. Les conditions d'hybridation appropriées correspondent aux conditions de température et de force ionique usuellement utilisées par l'homme du métier (Sambrook et al., 1989), de préférence à des

conditions de température comprises entre ( $T_m$  moins 5°C) et ( $T_m$  moins 30°C) et de préférence encore, à des conditions de température comprises entre ( $T_m$  moins 5°C) et ( $T_m$  moins 10°C) (forte stringence),  $T_m$  étant la température théorique de fusion, définie comme étant la température à laquelle 50 % des brins appariés se séparent. De telles sondes font également partie de l'invention. Elles peuvent être utilisées comme outil de diagnostic *in vitro* pour la détection, par des expériences d'hybridation, de transcrits spécifiques des polypeptides de l'invention dans des échantillons biologiques ou pour la mise en évidence de synthèses aberrantes ou d'anomalies génétiques résultant d'un polymorphisme, de mutations ou d'un mauvais épissage.

Les sondes de l'invention comportent au minimum 10 nucléotides, et au maximum comportent la totalité d'une séquence nucléotidique choisie parmi SEQ ID n° 1, n° 3, n° 5 et n° 7 ou de leur brin complémentaire.

Les méthodes de diagnostic *in vitro* dans lesquelles ces sondes nucléotidiques sont mises en oeuvre pour la détection de synthèses aberrantes ou d'anomalies génétiques, telles que la perte d'hétérozygotie et le réarrangement génétique, au niveau des séquences nucléiques codant pour un polypeptide ULIP selon l'invention ou un fragment biologiquement actif, sont incluses dans la présente invention. Un tel type de méthode comprend :

- la mise en contact d'une sonde nucléotidique de l'invention avec un échantillon biologique dans des conditions permettant la formation d'un complexe d'hybridation entre ladite sonde et la susdite séquence nucléotidique, éventuellement après une étape préalable d'amplification de la susdite séquence nucléotidique ;



- la détection du complexe d'hybridation éventuellement formé ;
- éventuellement le séquençage de la séquence nucléotidique formant le complexe d'hybridation avec la sonde de l'invention.

Les sondes d'ADNc de l'invention sont en outre  
5 avantageusement utilisables pour la détection d'anomalies chromosomiques.

Les séquences nucléotidiques selon l'invention sont également utiles pour la réalisation et l'utilisation d'amorces oligonucléotidiques sens et/ou antisens pour des réactions de séquençage ou d'amplification spécifique selon la technique dite de PCR (réaction de polymérisation en  
10 chaîne) ou toute autre variante de celle-ci.

Les séquences nucléotidiques selon l'invention ont par ailleurs des utilisations dans le domaine thérapeutique, pour la réalisation de séquences antisens, capables de s'hybrider spécifiquement avec une séquence d'acide nucléique, y compris un ARN messenger, utilisables en  
15 thérapie génique. L'invention a ainsi pour objet des séquences antisens capables d'inhiber, au moins partiellement, la production d'un polypeptide selon l'invention, tel que défini précédemment.

Elles sont plus particulièrement utiles dans le traitement des désordres du système nerveux central et périphérique et de la vision,  
20 notamment dans le traitement des syndromes neurologiques paranéoplasiques, ainsi que dans le traitement anticancéreux, notamment des tumeurs associées à des syndromes neurologiques paranéoplasiques.

Les séquences nucléotidiques selon l'invention peuvent par ailleurs être utilisées pour la production de protéines recombinantes ULIP  
25 selon l'invention.

Ces protéines peuvent être produites à partir des séquences nucléotidiques définies ci-dessus, selon des techniques de production de

produits recombinants connues de l'homme du métier. Dans ce cas, la séquence nucléotidique utilisée est placée sous le contrôle de signaux permettant son expression dans un hôte cellulaire.

Un système efficace de production d'une protéine  
5 recombinante nécessite de disposer d'un vecteur, par exemple d'origine plasmidique ou virale, et d'une cellule hôte compatible.

L'hôte cellulaire peut être choisi parmi des systèmes procaryotes, comme les bactéries, ou eucaryotes, comme par exemple les levures, cellules d'insectes, CHO (cellules d'ovaires de hamster chinois)  
10 ou tout autre système avantageusement disponible. Un hôte cellulaire préféré pour l'expression des protéines de l'invention est constitué par la bactérie *E. coli*.

Le vecteur doit comporter un promoteur, des signaux d'initiation et de terminaison de la traduction, ainsi que les régions  
15 appropriées de régulation de la transcription. Il doit pouvoir être maintenu de façon stable dans la cellule et peut éventuellement posséder des signaux particuliers spécifiant la sécrétion de la protéine traduite.

Ces différents signaux de contrôle sont choisis en fonction de l'hôte cellulaire utilisé. A cet effet, les séquences nucléotidiques selon  
20 l'invention peuvent être insérées dans des vecteurs à réplication autonome au sein de l'hôte choisi, ou des vecteurs intégratifs de l'hôte choisi. De tels vecteurs seront préparés selon les méthodes couramment utilisées par l'homme du métier, et les clones en résultant peuvent être introduits dans un hôte approprié par des méthodes standard, telles que  
25 par exemple l'électroporation.

L'invention vise en outre les cellules hôtes transfectées par ces vecteurs précédents. Ces cellules peuvent être obtenues par

l'introduction dans des cellules hôtes d'une séquence nucléotidique insérée dans un vecteur tel que défini ci-dessus, puis la mise en culture desdites cellules dans des conditions permettant la réplication et/ou l'expression de la séquence nucléotidique transfectée.

5 Ces cellules sont utilisables dans une méthode de production d'un polypeptide recombinant selon l'invention ou tout fragment ou dérivé biologiquement actif de celui-ci.

La méthode de production d'un polypeptide de l'invention sous forme recombinante est elle-même comprise dans la présente  
10 invention, et se caractérise en ce que l'on cultive les cellules transfectées dans des conditions permettant l'expression d'un polypeptide recombinant selon l'invention ou de tout fragment ou dérivé biologiquement actif de celui-ci, et que l'on récupère ledit polypeptide recombinant.

Les procédés de purification utilisés sont connus de l'homme  
15 du métier. Le polypeptide recombinant peut être purifié à partir de lysats et extraits cellulaires, du surnageant du milieu de culture, par des méthodes utilisées séparément ou en combinaison, telles que le fractionnement, les méthodes de chromatographie, les techniques d'immunoaffinité à l'aide d'anticorps mono ou polyclonaux spécifiques,  
20 etc.

Une variante consiste à produire un polypeptide recombinant fusionné à une protéine "porteuse" (protéine chimère). L'avantage de ce système est qu'il permet une stabilisation et une diminution de la protéolyse du produit recombinant, une augmentation de  
25 la solubilité au cours de la renaturation *in vitro* et/ou une simplification de la purification lorsque le partenaire de fusion possède une affinité pour un ligand spécifique.

L'exploitation des protéines ULIP, et en particulier POP-66/ULIP-4, ainsi que des anticorps dirigés contre ces protéines, est prometteuse dans divers domaines.

5           Ainsi, la détection de l'auto-anticorps anti-CV2 par immunofluorescence sur cerveau animal fixé est utilisée actuellement comme test diagnostic.

La production de protéine recombinante POP-66/ULIP-4 selon l'invention permet la fabrication d'un test (de type Elisa ou Western  
10   Blot) rapide et fiable, pour détecter les anticorps anti-CV2.

De tels tests existent déjà pour les anticorps anti-Hu, anti-Yo et anti-Ri. Le test pour détecter les anti-CV2 dans le sérum des patients pourrait être prescrit en cas de suspicion de syndrome neurologique paranéoplasique et inclurait par conséquent les anticorps anti-CV2 au  
15   même titre que les autres anticorps identifiés dans les SNP tels que précédemment cités.

L'invention vise donc également une méthode pour le diagnostic des syndromes neurologiques paranéoplasiques et/ou pour le  
20   diagnostic précoce de la formation de tumeurs d'origine cancéreuse, caractérisée en ce que l'on met en évidence dans un échantillon de sang prélevé chez un individu des auto-anticorps dirigés contre une protéine POP-66/ULIP-4 par

- la mise en contact un échantillon de sang prélevé chez un  
25   individu avec un polypeptide purifié (POP-66), dérivé ou fragment polypeptidique biologiquement actif de POP-66/ULIP-4 éventuellement fixé sur un support dans des conditions permettant la formation de

complexes immunologiques spécifiques entre ledit polypeptide et les auto-anticorps éventuellement présent dans l'échantillon de sérum, et

- la détection des complexes immunologiques spécifiques éventuellement formés.

5 L'invention a également pour objet un kit pour le diagnostic des syndromes neurologiques paranéoplasiques et pour le diagnostic précoce de la formation des tumeurs à partir d'un prélèvement biologique comprenant :

10 - au moins un polypeptide purifié POP-66/ULIP-4, dérivé ou fragment polypeptidique biologiquement actif de POP-66/ULIP-4, éventuellement fixé sur un support,

15 - des moyens de révélation de la formation de complexes antigène/anticorps spécifiques entre un auto-anticorps anti-POP-66 et ledit polypeptide purifié POP-66, dérivé ou fragment polypeptidique et/ou des moyens de quantification de ces complexes.

20 L'invention a également pour objet les anticorps mono- ou polyclonaux ou leurs fragments, anticorps chimériques ou immunoconjugués, obtenus à partir d'un polypeptide purifié ULIP comprenant une séquence d'acides aminés choisie parmi SEQ ID n° 2, n° 4, n° 6 et n° 8, dérivé ou fragment polypeptidique biologiquement actif de ULIP et leur utilisation, pour la purification ou la détection d'une protéine ULIP dans un échantillon biologique.

25 Des anticorps polyclonaux peuvent être obtenus à partir du sérum d'un animal immunisé contre la protéine, produite par exemple par recombinaison génétique suivant la méthode décrite ci-dessus, selon les modes opératoires usuels.

Les anticorps monoclonaux peuvent être obtenus selon la méthode classique de culture d'hybridomes décrite par Köhler et Milstein.

Les anticorps peuvent être des anticorps chimériques, des anticorps humanisés, des fragments Fab et F(ab')<sub>2</sub>. Ils peuvent  
5 également se présenter sous forme d'immunoconjugués ou d'anticorps marqués.

L'invention porte également sur l'utilisation d'anticorps dirigés contre une protéine de la famille ULIP pour la mise en évidence  
10 d'une protéine ULIP dans des néoplasmes, et des syndromes neurologiques paranéoplasiques à des fins de diagnostic.

De manière préférentielle, l'invention porte sur l'utilisation d'anticorps monoclonaux obtenus à partir du sérum polyclonal anti-CV2 de patients par immortalisation de lymphocytes, selon les techniques  
15 usuelles connues de l'homme du métier.

Ainsi, les anticorps dirigés contre une protéine de la famille ULIP sont utiles pour détecter une expression anormale de protéine ULIP chez des patients présentant des syndromes neurologiques, chez qui aucun cancer n'a été diagnostiqué par les méthodes classiques. Cette  
20 expression anormale de protéine ULIP pourra être corrélée à l'existence d'un cancer qui n'avait pas été repéré. Ainsi, les anticorps dirigés contre une protéine ULIP, notamment contre POP-66/ULIP-4, sont utiles pour le diagnostic précoce d'un cancer.

25 L'invention a également pour objet une méthode de détermination d'une variabilité allélique, d'une mutation, d'une délétion, d'une insertion, d'une perte d'hétérozygotie ou d'une anomalie génétique

du gène POP-66/ULIP-4, situé sur le chromosome 10 dans la région 26q pouvant être impliquées dans des pathologies, caractérisée en ce qu'elle met en oeuvre au moins une séquence nucléotidique SEQ ID n° 7. Parmi les méthodes de détermination d'une variabilité allélique, d'une mutation, d'une délétion, d'une insertion, d'une perte d'hétérozygotie ou d'une anomalie génétique du gène POP-66/ULIP-4, on préfère une méthode comprenant au moins une étape d'amplification par PCR de la séquence nucléique de POP-66/ULIP-4 susceptible de présenter un polymorphisme. une mutation, une délétion ou une insertion, à l'aide de couple d'amorces de séquences nucléotidiques, une étape au cours de laquelle on procède au traitement des produits amplifiés à l'aide d'enzymes de restriction appropriées et une étape au cours de laquelle on procède à la détection ou au dosage d'au moins l'un des produits de la réaction enzymatique.

De manière avantageuse, on peut rechercher les mutations associées audit chromosome 10 en relation avec le cancer, notamment les tumeurs cancéreuses périphériques et les tumeurs cérébrales primitives d'origine gliale par exemple.

L'invention a également pour objet une composition pharmaceutique comprenant au moins une protéine purifiée de la famille ULIP, fragment polypeptidique ou dérivé biologiquement actif de celle-ci, une séquence ou fragment de séquence nucléotidique codant pour ladite protéine, une séquence anti-sens capable de s'hybrider spécifiquement avec une séquence nucléotidique codant pour ladite protéine, ou un anticorps dirigé contre ladite protéine, associée à un véhicule pharmaceutiquement acceptable.

L'invention comprend de manière préférentielle des compositions pharmaceutiques comprenant comme principe actif un polypeptide POP-66 purifié, dérivé ou fragment polypeptidique de POP-66, préférentiellement sous forme soluble, associé à un véhicule pharmaceutiquement acceptable.

De telles compositions offrent une nouvelle approche pour traiter les désordres du système nerveux central et périphérique et de la vision, et notamment les syndromes neurologiques paranéoplasiques. Par ailleurs, elles sont utiles pour traiter les désordres neurologiques liés à une perte neuronale et/ou une sous-expression des protéines ULIP dans le système nerveux.

Ainsi, POP-66/ULIP-4 révèle aussi un intérêt dans des pathologies neurodégénératives telles que les atrophies multisystémiques qui sont des affections similaires à celles des SNP et pour lesquelles une anomalie d'une sous-population oligodendrocytaire a été détectée (Papp et al., 1992).

Les compositions selon l'invention sont par ailleurs utiles en thérapie anticancéreuse.

Les anticorps dirigés contre une ou plusieurs protéines ULIP peuvent être associés à des agents antinéoplasiques, permettant ainsi le ciblage des médicaments vers les cellules tumorales.

Ils peuvent en outre être associés à un groupe chimique hydrophile choisi de manière à passer ou non la barrière hémato-encéphalique, selon le type de tumeur.

Les protéines ULIP et en particulier POP-66 ainsi que les séquences nucléotidiques codant pour lesdites protéines et les séquences ou oligonucléotides anti-sens, peuvent être utiles dans la



thérapie de tout type de cancer dans lequel un gène codant pour une protéine ULIP est impliqué. Parmi des exemples de cancers, on peut citer les tumeurs périphériques, telles que le cancer du poumon à petites cellules, le thymome, le cancer du sein et de l'ovaire, ainsi que les  
5 tumeurs cérébrales, de préférence les tumeurs cérébrales primitives d'origine gliale. L'expression de POP-66 dans les cellules non prolifératives du cerveau normal, son absence dans des tissus normaux tels que poumon ou thymus par exemple, sa réexpression différentielle lors de la tumorigénèse de ces tissus et la modulation de son expression  
10 dans une lignée tumorale au cours de la différenciation suggèrent à cet égard que POP-66 pourrait être un gène suppresseur de tumeur.

Préférentiellement, les compositions pharmaceutiques selon l'invention peuvent être administrées par voie systémique, de préférence par voie intraveineuse, par voie intramusculaire, intradermique ou par  
15 voie orale.

Leurs modes d'administration, posologies et formes galéniques optimaux peuvent être déterminés selon les critères généralement pris en compte dans l'établissement d'un traitement thérapeutique adapté à un patient comme par exemple l'âge ou le poids  
20 corporel du patient, la gravité de son état général, la tolérance au traitement et les effets secondaires constatés, etc.

L'invention comprend également l'utilisation d'une protéine purifiée de la famille ULIP, fragment polypeptidique ou dérivé  
25 biologiquement actif de celle-ci, une séquence ou fragment de séquence nucléotidique codant pour ladite protéine, une séquence anti-sens capable de s'hybrider spécifiquement avec une séquence nucléotidique

codant pour ladite protéine, ou un anticorps dirigé contre ladite protéine, associée à un véhicule pharmaceutiquement acceptable, pour la fabrication d'un médicament destiné à traiter les maladies neurodégénératives et les néoplasmes.

5

L'invention a enfin pour objet une méthode de traitement des maladies neurodégénératives et des néoplasmes comprenant l'administration à un sujet nécessitant un tel traitement d'une quantité thérapeutiquement efficace d'une protéine purifiée de la famille ULIP, 10 fragment polypeptidique ou dérivé biologiquement actif de celle-ci, une séquence ou fragment de séquence nucléotidique codant pour ladite protéine, une séquence anti-sens capable de s'hybrider spécifiquement avec une séquence nucléotidique codant pour ladite protéine, ou un anticorps dirigé contre ladite protéine, associée à un véhicule 15 pharmaceutiquement acceptable.

Les exemples et les figures dont les légendes sont présentées ci-après sont donnés à titre illustratif.

20

### **LEGENDE DES FIGURES**

- La figure 1 représente un profil d'électrophorèse à deux dimensions obtenu à partir d'extraits protéiques de cerveaux de rats 25 nouveaux-nés enrichis en POP-66.

A : coloration à l'argent de l'ensemble des protéines.

B : immunoempreinte avec le sérum anti-CV2 de patients.

Les flèches indiquent les taches correspondant à POP-66, révélées avec les anticorps anti-CV2.

- La figure 2 représente un profil d'électrophorèse à deux dimensions obtenue à partir d'extraits protéiques de cerveaux de rats nouveaux-nés.

Immunoempreinte avec A- l'anticorps antipeptide 3 et B- l'anticorps anti-CV2.

- La figure 3 représente une électrophorèse à une dimension obtenue à partir d'extraits protéiques de cerveaux de rats nouveaux-nés.

Immunoempreinte avec a : sérum préimmun pour peptide 3

Immunoempreinte avec b : sérum anti-peptide 3

Immunoempreinte avec c : sérum anti-peptide 4

Immunoempreinte avec d : sérum préimmun pour peptide 4.

- La figure 4 représente un marquage immunohistochimique de coupes de cerveaux de rats adultes avec

A : sérum anti-CV2 de patient atteint de SNP

B : sérum de lapin avec des anticorps anti-peptide 3

C : sérum de lapin avec des anticorps anti-peptide 4.

- La figure 5 représente un marquage histologique de coupes de cervelet de raton à 8 jours post-natal.

A : coloration au bleu de toluidine ; ge = couche granulaire externe ; m = couche moléculaire (x400).

B : Immunomarquage après incorporation de BrdU (bromodéoxyuridine). Les cellules ayant incorporé le BrdU sont pratiquement toutes situées dans la zone la plus externe de la couche granulaire externe (ge). Quelques cellules positives sont situées dans la couche granulaire interne (x400).

C : Immunoperoxydase indirecte avec un sérum de patient contenant un anticorps anti-CV2 (x400). L'immunoréactivité est concentrée dans la partie interne de la couche granulaire externe (future couche moléculaire (m)). Quelques cellules sont immunoréactives dans la  
5 couche granulaire interne. Les cellules de Purkinje (p) sont négatives ainsi que les cellules de la partie externe de la couche granulaire externe (ge).

D : Immunoperoxydase indirecte avec un sérum de patient contenant un anticorps anti-CV2 (x1000). L'immunomarquage est surtout  
10 concentré dans la partie interne de la couche granulaire externe (future couche moléculaire (m)). On note une cellule réactive dans la couche granulaire interne (gi) (flèche).

- La figure 6 représente un marquage immunohistochimique de coupes d'hippocampe humain post-mortem (coloration HPS).

15 A : cerveau de patient témoin,

B : cerveau de patient présentant une encéphalite limbique, et anticorps circulant anti-CV2. On peut noter la disparition des cellules granulaires.

- La figure 7 représente un profil d'électrophorèse à deux  
20 dimensions avec la protéine ULIP-2 (A) contrôle et la protéine ULIP-4 (B).

La figure 7C représente le modèle de profil de migration des protéines ULIP-1, 2, 3 et 4 comme référence.

Les protéines sont révélées :

a) par autoradiographie pour localiser les protéines traduites  
25 *in vitro* (traduction) ;

b) par immunoempreinte avec le sérum anti-CV2.

- La figure 8 représente un profil de migration de l'ARNm de C-22/ULIP-3 (8A) et TOAD-64/ULIP-2 (8B) amplifié par RT-PCR exprimé dans différents types cellulaires :

pistes 1-3 : tumeur à petites cellules du poumon

5        piste 2 : tumeur à petites cellules du poumon avec du sérum anti-CV2

piste 4 : cDNA contrôle.

piste 5 : médulloblastome traité par infection HTLV1

pistes 6-7 : médulloblastome

10        piste 8 : lignée C6 de cellules gliales chez la souris

piste 9 : contrôle

piste 10 : néant

piste 11 : échelle kb.

15        Les flèches noires correspondent à POP-66, les flèches blanches correspondent au standard de poids moléculaire.

- La figure 9 représente la séquence nucléotidique d'ULIP-2 chez la souris (SEQ ID N° 1), ainsi que la séquence en acides aminés déduite (SEQ ID N° 2).

20        - La figure 10 représente la séquence nucléotidique d'ULIP-3 chez la souris (SEQ ID N° 3), ainsi que la séquence en acides aminés déduite (SEQ ID N° 4).

- La figure 11 représente la séquence nucléotidique d'ULIP-4 chez la souris (SEQ ID N° 5), ainsi que la séquence en acides aminés déduite (SEQ ID N° 6).

25        - La figure 12 représente la séquence nucléotidique d'ULIP-4 chez l'homme (SEQ ID N° 7), ainsi que la séquence en acides aminés déduite (SEQ ID N° 8).

Un codon stop erroné dans la séquence ULIP-4 homme (astérisque) provient d'une faute de la transcriptase inverse dans la production de la banque. En comparant avec ULIP-4 de rat et de souris, il est presque certain que la séquence TAG codant pour un stop soit en fait  
5 un codon AAG, codant pour une lysine comme chez le rat et la souris. De plus, la région autour de cet acide aminé est entièrement conservée dans les trois espèces.

La séquence d'acides aminés a été complétée sur la SEQ ID n° 8 par 15 acides aminés en C-terminal (n° 554 à n° 568). Cette région  
10 C-terminale manquante sur la figure 12 est très bien conservée entre ULIP-4 rat et souris ainsi qu'entre les différentes ULIP.

### **EXEMPLE 1 :**

#### **Purification de POP-66 et séquençage**

La purification de POP-66 est réalisée selon le matériel et les méthodes décrits dans l'article de Honnorat et al., 1996, incorporé par référence, à partir de sérum de patients atteints de SNP.

20 Pour identifier la protéine POP-66, on a choisi une stratégie de purification qui permette d'obtenir un séquençage partiel. Le criblage d'une banque d'expression d'ADNc de cerveau ou la purification de la protéine par immunoaffinité étaient exclus en raison des quantités limitées de sérums liées au décès des patients. Une méthode de purification  
25 biochimique a pu être développée à partir de cerveaux de rats nouveaux-nés grâce aux sérums humains anti-CV2 qui ont permis de suivre chaque étape de purification.

Les tissus, conservés à  $-70^{\circ}\text{C}$  avant utilisation, ont été traités par une solution contenant DTT (dithiothreitol) (Sigma) 0,2 M, Ampholine 3-10 (Pharmacia) 2 %, Triton X-100 (Merck) 2 % et placés à  $2-4^{\circ}\text{C}$ . Immédiatement avant l'utilisation, de l'urée solide (Pharmacia) a été ajoutée pour obtenir une solution 8M.

La protéine POP-66 est soluble, au moins en partie, et précipite entièrement à une concentration de 40 % de sulfate d'ammonium.

Une centrifugation à 100 000 (fois) g et une précipitation au sulfate d'ammonium (éliminant les protéines précipitant en-dessous de 20 % et au-dessus de 40 % de sulfate d'ammonium) permettent d'obtenir des extraits protéiques enrichis en POP-66. Les protéines de cet extrait sont alors séparées, après dialyse, par isofocalisation sur gel d'agarose (Peltre et al., 1982).

Après transfert sur membrane, les anticorps anti-CV2 reconnaissent plusieurs bandes de points isoélectriques compris entre 5,85 et 6,55. L'ensemble de ces bandes correspond à la protéine POP-66 reconnue par les anticorps anti-CV2. Ce spectre suggère la possibilité de modifications transcriptionnelles (phosphorylations et/ou glycosylations) de la protéine.

A partir du gel d'agarose, la zone des protéines comprise entre 5,85 et 6,55, de pI, est utilisée pour une nouvelle migration électrophorétique en milieu dénaturant sur gel de polyacrylamide préalablement équilibré avec une solution d'équilibration (0,05 mol/l Tris/HCl, pH 6,8, urée 6M, glycérol 30 %, SDS 1 % poids/volume pendant  $2 \times 10$  minutes) à laquelle on ajoute du DTT (0,25 % poids /volume) et du bleu de bromophénol.

Deux modes de détection sont utilisés :

- *la coloration à l'argent*. Immédiatement après la fin de la migration, le gel est immergé dans une solution fixatrice (40 % d'éthanol, 10 % d'acide acétique) pendant 30 minutes ; il est ensuite placé dans une solution d'incubation (30 % d'éthanol, 7 % poids/volume d'acétate de sodium, 0,1 % de glutaraldéhyde, 0,2 % poids/volume de thiosulfate de sodium) pendant 30 minutes ou une nuit. Après lavage, le gel est placé dans une solution d'argent (0,1 % poids/volume de nitrate d'argent + formaldéhyde) et développé (2,5 % poids/volume de carbonate de sodium + formaldéhyde). La réaction est arrêtée avec l'EDTA- $\text{Na}_2$  (1,5 % poids/volume). Les gels sont conservés dans une solution de glycérol.

- *transfert sur membrane de PVDF* (Immobilon-P®, Millipore). Les protéines séparées sont transférées sur une membrane de PVDF en utilisant un tampon CAPS (Sigma) 100 mM pH 11. Les transferts sont incubés pendant une heure dans du tampon TBS (Tris buffer saline) avec 5 % de caséine (lait) et 18 heures dans du tampon TBS (+ 1 % de caséine) contenant de l'anticorps (sérum anti-CV2 1/500). Après lavage avec TBS-caséine (15 minutes), la révélation est effectuée en incubant les transferts pendant 1 heure et demie avec les anticorps anti-IgG biotinylés (1/1000) et pendant 1 heure et demie avec le complexe streptavidine-peroxydase (1/2000). Les transferts sont ensuite révélés avec du DAB (diaminobenzidine 0,06 % poids /volume dans Tris 0,05 M) et avec  $\text{H}_2\text{O}_2$  (0,02  $\mu\text{g}/\text{ml}$ ).

Une seule bande correspondant à une protéine de 66 kDa est visible. Celle-ci est spécifiquement marquée par les anticorps anti-CV2 (figure 1). On a effectué alors un séquençage N-terminal de cette protéine, après digestion trypsique.



Sept peptides, présentant les séquences suivantes, ont été obtenus :

- 1 - X-Met-Tyr-Asp-Gly-Pro
  - 2 - X-Phe-Asn-Leu-Tyr-Pro-Arg
  - 5 3 - X-Val-Leu-Glu-Asp-Gly-Thr-Leu-His-Val-Thr-Glu-Gly
  - 4- X-Ile-Gly-X-X-Ala-Gln-Val-(His ?)-Ala-Glu-Asn-Gly-X-Ile-Ile-Ala-Glu-Glu-Gln
  - 5 - X-X-Glu-Asn-Gln-Phe-Val-Ala-Val-Thr
  - 6 - X-Val-Asn-Asp-(Asp ?)-Gln-Ser-Phe-Tyr-Ala-Asp-Ile-Tyr-Met-Glu-
  - 10 (Asp ?)-(Gly ?)-Leu-Ile
  - 7 - X-X-X-Phe-Val-Thr-X-Pro-X-Leu-X-Pro
- X : correspond à un acide aminé non déterminé,  
( ? ) : correspond à un acide aminé probable mais non certain.

15 D'après l'analyse des banques de données disponibles en 1994, aucune protéine connue ne correspondait à ces séquences.

### **EXEMPLE 2 :**

20 **Clonage de l'ADNc de POP-66 ou des protéines apparentées**

Le clonage de l'ADNc de la protéine POP-66 ou des protéines apparentées a été entrepris en utilisant des sondes  
25 oligonucléotidiques dégénérées obtenues à partir de fragments de deux peptides :

Ile-Ile-Ala-Glu-Glu-Gln

Tyr-Ala-Asp-Ile-Tyr-Met-Glu-(Asp ?)

Quatre jeux d'amorces oligonucléotidiques dégénérées  
5 (sens/anti-sens) sont donc déterminés

(AT(C/T)ATTGC(T/A)GA(A/G)CA ; TG(C/T)TC(T/C)AC(T/A)GCAT(A/G)AT;  
TATGC(A/T)GA(C/T)AT(C/T)ATGGA ; TCCAT(G/A)TA(G/A)CT(T/A)GCAT  
A) et utilisés pour une amplification PCR.

La matrice est préparée sous forme d'ADNc double-brin  
10 (Promega kit) à partir d'ARNpoly(A\*) extrait du cerveau de rats âgés de 10  
jours (Zivic-Miller, USA) en utilisant le kit d'isolement de l'ARNm Fast  
Track (Invitrogen).

Les conditions d'amplification par PCR sont les suivantes :  
35 cycles à 94°C, 1 minute pour la dénaturation, 55°C, 1 minute pour  
15 l'hybridation et 72°C, deux minutes pour l'extension.

Les produits de PCR sont analysés en électrophorèse sur  
gel d'agarose à 1 %, électroélués, clonés dans un vecteur de clonage TA  
(Invitrogen) et séquencés en utilisant les sites des amorces des  
promoteurs T7 et SP6.

20 La séquence d'acides aminés déduite du clone MFB-17  
concorde avec les séquences des deux peptides originaux de POP-66  
déterminés par l'analyse de la séquence d'acides aminés.

Une analyse comparée des séquences d'acides nucléiques  
utilisant les bases de données Genbank et EMBL révèle que MFB-17 est  
25 un ADNc partiel avec une séquence nucléotidique identique à celle d'un  
segment de TOAD-64, une protéine neuronale de rat (Minturn et al.,  
1995).

La séquence d'acides aminés déduite à l'ADNc de TOAD-64 concorde avec les séquences des sept peptides déterminés par l'analyse des séquence partielles de la protéine reconnue par les anticorps anti-CV2 après purification par électrophorèse.

5 Le poids moléculaire, le point isoélectrique, le profil immunohistochimique et la régulation de TOAD-64 sont similaires à ceux de l'antigène POP-66.

Le clone MFB-17 ne présentant pas la région codante complète, il a été nécessaire de produire une protéine recombinante  
10 intacte pour poursuivre les recherches concernant la protéine CV2.

Pour obtenir une protéine complète TOAD-64, on a amplifié la matrice ADNc-ds de cerveaux de rats avec deux jeux d'amorces situées aux extrémités 5' et 3' des régions codantes  
(sens : GGCATATGTCTTATCAGGGGAAG ;  
15 anti-sens GCGAATTCTTAGCCCAGGCTGATG).

Cette approche a permis de produire deux clones différents, l'un correspondant à la séquence TOAD-64 et l'autre à un clone désigné par C-22.

20

### **EXEMPLE 3 :**

#### **Comparaison de la séquence d'acides aminés déduite de C-22 avec les protéines ULIP**

La séquence d'acides aminés déduite à partir du cadre de lecture ouverte, indique que ce clone C-22 appartient à la super-famille  
25 de gènes ULIP représentée par plusieurs gènes des séquences EST.

La séquence d'acides aminés déduite de C-22 présente une homologie de 30 % avec la séquence d'acides aminés de la protéine unc-33 de *Caenorhabditis elegans*.

5 Récemment, quatre gènes différents homologues à la protéine unc-33 ont été décrits chez les mammifères et le poulet.

Une analyse des séquences par les bases de données Genbank et banques de protéines a permis de proposer une classification des protéines unc-33 like (ULIP) en quatre différents sous-groupes (Byk  
10 et al. 1996).

Pourtant, comme les fonctions réelles de ces protéines ne sont pas clairement connues, la classification proposée est basée simplement sur le pourcentage d'identité d'acides aminés. ULIP-1 est représentée par une phospho-protéine « unc-33 like » de souris qui  
15 présente une homologie de 76 % avec TOAD-64, Crmp-62, et Munc, une séquence de souris récemment disponible sur Genbank.

ULIP-2 est composée de TOAD-64, Crmp-62 et Munc qui présentent entre eux une identité de 97 % d'acides aminés.

Les séquences partielles humaines EST, c'est-à-dire hcrmp-  
20 1, qui présentent une identité de 75 % avec ULIP-1 ou ULIP-2 ont été trouvées. Elles appartiennent à un troisième groupe appelé ULIP-3. Le dernier groupe identifié appelé ULIP-4, comprend r-CRMP-3 chez le rat et les formes ULIP-4 chez la souris et POP-66/ULIP-4 chez l'homme.

La comparaison de la séquence d'acides aminés des trois  
25 gènes ULIP, à savoir TOAD-64 chez le rat, Crmp-62 chez le poulet, et ULIP-1 chez la souris, avec la séquence d'acides aminés déduite du cadre de lecture ouvert du présent clone C-22, en utilisant le logiciel

d'alignement Clustal V, révèle que C-22 présente une identité de 74% avec ULIP-1, 77 % avec Crmp-62 et 76 % avec TOAD-64.

La séquence nucléotidique C-22 a une identité de 97 % avec la séquence partielle EST, hCrmp-1, et définit ainsi le troisième  
5 membre du groupe ULIP-3. Les gènes TOAD-64, Crmp-62 et C-22 codent chacun pour une protéine de 572 acides aminés de longueur, tandis que la séquence d'acides aminés déduite à partir d'ULIP-1 donne une protéine de 570 acides aminés.

L'analyse de la séquence d'acides aminés de C-22 ne  
10 montre aucune séquence de signal ou de domaine transmembranaire suggérant que le ou les produits du gène C-22 pourraient être localisés dans le cytoplasme des cellules.

Plusieurs sites consensus de phosphorylation par la protéine kinase C (S/T X R/K) apparaissent le long du produit du gène C-22. Ces  
15 observations suggèrent que C-22 est une phospho- protéine et que de légères différences dans la phosphorylation pourraient dicter l'activité ou le rôle des différents membres de cette famille de protéines tout au long du cycle cellulaire.

Tableau 1 : Récapitulation des protéines présentant une homologie avec les ULIP.

Famille		Espèces	N° EMBL
Unc-33 nematode		Nematode	Z14146
Dihydropyrimidinase	Hu DHPase	humain	D78011
	Ra DHPase	rat	D63704
groupe ULIP-1	Ulip	souris	X87817
	Hu DRP3	humain	D78014
	r-CRMP-1	rat	U52102
	Hu-Ulip	humain	Y07818
groupe ULIP-2	ULIP-2	souris	SEQ ID n° 2
	Toad-64	rat	Z46882
	CRMP-62	poulet	U17277
	Munc	souris	X87242
	HCRMP-2	humain	U17279
	Hu DRP-2	humain	D78013
	r-CRMP-4	rat	U52104
groupe ULIP-3	ULIP-3	souris	SEQ ID n° 4
	HCRMP-1	humain	U17278
	rCRMP-1	rat	U52102
	C-22	rat	U52095
	Hu DRP-1	humain	D78012
groupe ULIP-4	ULIP-4	souris	SEQ ID n° 6
	POP-66/ULIP-4	homme	SEQ ID n° 8
	r-CRMP-3	rat	U52103

**EXEMPLE 4 :****Régulation de l'expression du gène C-22 :**

L'évaluation des altérations dans l'expression du gène C-22  
5 pourrait avoir une importance considérable pour la connaissance des aspects fonctionnels de la protéine C-22.

Par conséquent, la Demanderesse a étudié la possible  
régulation de l'expression du gène C-22 au cours du développement.  
L'ARN total est extrait et séparé par l'électrophorèse sur gel d'agarose à  
10 1 % et transféré sur membrane Nytran (Duchemin et al. 1987). Les transferts sont hybridés avec une séquence codante C-22 marquée au <sup>32</sup>P, un tampon phosphate 0,5 mM et 5 % de SDS à 65°C pendant 16 heures.

A la fin de l'hybridation, les transferts sont lavés  
15 successivement trois fois avec 2xSSC, 0,1 % SDS à température ambiante, puis 1xSSC, 0,1 % SDS à 65°C pendant 60 minutes, et exposés aux rayons X.

Dans les conditions utilisées, on a détecté une seule bande  
à 3,8 kb représentant l'ARNm C-22 qui est aussi le plus petit transcrit de  
20 la famille de gènes unc-33 des vertébrés. La taille du transcrit reste la même durant les périodes pré et post-natales.

La cinétique du gène C-22 dans le cerveau de rats au cours  
du développement montre que le messager est détectable au cours de la  
période embryonnaire au jour E17. La quantité de transcrits C-22  
25 augmente jusqu'au jour 7 post-natal puis décroît rapidement à partir de la seconde semaine après la naissance jusqu'à un niveau pratiquement indétectable chez l'adulte.

Aux environs de la naissance, un signal de régulation encore inconnu est probablement reçu, ce qui augmente l'expression du gène C-22, ce signal étant lié temporairement à la différenciation neuronale et au développement axonal.

5 L'ARNm de C-22 n'a pas pu être détecté par analyse Northern Blot dans plusieurs régions du cerveau telles que le cortex frontal, le cerveau moyen et le thalamus chez l'adulte et le rat âgé de plus de deux ans.

De plus, on n'a pas pu détecter l'ARNm de C-22 dans des  
10 tissus non neuronaux, tels que le coeur, le poumon, le foie, le rein chez des rats d'une semaine et des rats adultes.

Les données sur le profil de l'expression l'ARNm de C-22 suggèrent un rôle prépondérant de la protéine C-22 dans le développement du cerveau.

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### **EXEMPLE 5 :**

#### **Immunoempreinte de POP-66 :**

20

#### **A - Matériels et méthodes**

##### **• Transfection des ULIP dans *E. coli***

Les ADNc de pleine longueur d'ULIP-2 et ULIP-3 de rat et ULIP-1 et ULIP-4 de souris ont été sous-clonées de manière  
25 directionnelle dans le vecteur d'expression pET-21a(+) *E. Coli* après introduction d'un site 5'Nde I et d'un site 3' EcoRI par PCR, et les quatre constructions ont été reséquencées. L'expression de gène cible induit par IPTG a été réalisée selon le protocole du fabricant (Novagen).



- **Production d'anticorps anti-ULIP**

Des anticorps de lapin (anti-Pep3) sont dirigés contre le peptide ITGPEGHVLSRP EEVE (acides aminés 217-232 de la séquence SEQ ID n° 8), synthétisé sur un appareil de synthèse peptidique multiple utilisant le F-moc (432A Peptide Synthesizer SYNERGY, Applied Biosystems). La pureté a été vérifiée par analyse de la séquence par HPLC et spectrométrie de masse. 1 mg du peptide synthétique conjugué à de l'hémocyanine de patelle, dans de l'adjuvant complet de Freund, a été utilisé pour immuniser des lapins avec une dose « booster » de 0.5 mg de peptide lié dans de l'adjuvant complet de Freund après 4 semaines. Les anticorps anti-Pep3 ont reconnu les quatre protéines ULIP recombinantes exprimées dans *E. Coli*.

Le marquage avec les anticorps anti-Pep3 a été éliminé après préincubation avec le peptide 3. Des contrôles avec des sérums de lapin pré-immuns étaient négatifs.

Des anticorps anti-peptide 4 dirigés contre le peptide LEDGTLHVTEGS ont été produits selon le même protocole.

## **B - Résultats**

Des anticorps contre quatre des peptides séquencés ont été produits. Deux des sérums se sont avérés particulièrement intéressants.

L'un contient des anticorps (Ac anti-pep3) qui reconnaissent plusieurs membres de la famille ULIP en électrophorèse à deux dimensions d'extraits protéiques de cerveau de rat nouveau né (figure 2) et sur électrophorèse à une dimension (figure 3). Un autre anticorps (Ac

anti-pep4) reconnaît en Western Blot une seule bande de 66 kDa susceptible de correspondre à un seul membre de la famille (figure 3), à savoir ULIP-2.

5

#### **EXEMPLE 6 :**

##### **Immunohistochimie**

Les préparations de tissus pour l'immunohistochimie sont  
10 obtenues à partir de cerveaux de rats nouveaux-nés et de cerveaux humains *post-mortem*, fixés à 4° C dans du paraformaldéhyde à 4 % et 0,2 % d'acide picrique dilués dans du tampon phosphate (0,1 M, pH =7,4), puis cryoprotégés.

L'immunocytochimie peut être réalisée par la technique  
15 d'immunofluorescence indirecte. Des coupes de 12 µm d'épaisseur sont réalisées au cryostat puis montées sur lame recouverte de gélatine, traitées pendant 2 heures dans du tampon PBS et 1 % de sérumalbumine bovine (BSA) avec 0,1 % de Triton X100 et incubées 12 h avec le sérum de patients anti-CV2 dans du PBS-1% BSA à température ambiante  
20 (dilution du sérum 1/100). Après plusieurs lavages avec PBS-1 % BSA, les coupes sont incubées pendant 2 h avec un anti-sérum anti-humain de lapin conjugué à la fluorescéine diluée à 1 % (Dakopatts) dans du PBS-1 % BSA. Après lavage dans le PBS les lames sont examinées au microscope. Les coupes contrôle sont incubées avec soit l'antisérum IgG  
25 anti-humain conjugué à la fluorescéine seule, soit le PBS-1 % BSA seul, soit le sérum de patient seul, soit enfin le sérum contrôle (patients non

atteint de SNP) et anticorps conjugué à la fluorescéine à la même dilution.

Pour confirmer l'immunofluorescence on peut utiliser le marquage indirect par immunoperoxydase. Les coupes de tissus congelés  
5 fixés au paraformaldéhyde sont incubées avec 0,3 % d'H<sub>2</sub>O<sub>2</sub> (pour détruire l'activité peroxydase intrinsèque) et 10 % de sérum normal de lapin (pour éviter la liaison non spécifique des IgG de lapin) ou 1 % BSA. Après incubation 12 h avec des sérums de patients dilués à 1/1000 et lavage, les coupes sont incubées 2 h avec de l'antisérum IgG anti-humain  
10 de lapin biotinylé dilué à 1/1000 dans PBS-1% BSA. Les IgG humains liés sont visualisés par incubation avec un complexe avidine-biotine-peroxydase (Vectastain ABC complex, Vector) et développés avec 0.05 % DAB (Sigma). Les coupes contrôle sont obtenues avec des sérums de 15 patients sans SNP selon le même protocole.

15

#### **A - Localisation des protéines de la famille ULIP à l'aide d'anticorps anti-peptides :**

Un marquage immunohistochimique a été réalisé sur coupe de cerveaux de rats nouveaux-nés et adultes. L'anticorps anti-peptide-3  
20 reconnaît un (des) antigènes présent(s) dans plusieurs types cellulaires sur coupe de cerveaux de rats nouveau-nés et adultes (fig. 4). Comme le sérum anti-CV2 de patient, les anticorps anti-peptide-4 ne permettent la mise en évidence d'aucun antigène sur coupe de cerveau de rat nouveau né alors qu'ils marquent spécifiquement une sous-population  
25 d'oligodendrocytes dans le cerveau de rat adulte (fig. 4).

**B - Expression de POP-66 au cours du développement normal du cerveau :**

La figure 5 montre que les cellules nerveuses prolifératives des zones progénitrices du système nerveux mises en évidence par l'accumulation de bromodéoxyuridine (BrdU) n'expriment pas POP-66 alors que les cellules non prolifératives qui correspondent aux cellules nerveuses en différenciation ou en migration l'expriment.

**EXEMPLE 7 :**

**Rôle de POP-66 dans la survie neuronale**

La figure 6 permet de comparer des coupes de cerveaux humains de patients sains et de patients atteints de SNP. Chez les patients atteints d'un SNP et présentant des anticorps circulant anti-CV2, on observe une disparition des neurones du gyrus dentatus et des neurones pyramidaux (bande de cellules centrale), ainsi qu'une réaction astrocytaire intense.

**EXEMPLE 8 :**

**Caractérisation de la protéine POP-66 - Identification avec ULIP-4 :**

**Matériels et méthodes**

**a) Purification partielle d'ULIP-1**

ULIP-1 partiellement purifiée a été obtenue à partir de cerveaux de souris nouveaux-nés par trois étapes de purification. Ces cerveaux ont été homogénéisés dans 4 volumes de tampon d'homogénéisation (25 mM phosphate de sodium, pH 7,8, 1 mM EGTA, 10 µg/ml de leupeptine, 25 µg/ml d'aprotinine, et 10 µg/ml de pepstaine. Les homogénats ont été centrifugés pendant 10 minutes à 400 x g. Les culots ont été resuspendus dans 2 volumes de tampon d'homogénéisation, homogénéisés, et de nouveau centrifugés. Les surnageants issus des deux centrifugations ont été rassemblés, soniqués et centrifugés pendant 1 heure à 100.000 x g. Le surnageant (S2) a été chargé sur une colonne CL-6B de DEAE-Sepharose (1,75 cm<sup>2</sup> x 26 cm) équilibré avec 100 ml de tampon A (25 mM de phosphate de sodium, pH 7,8, 1 mM d'EGTA) à une flux de 30 ml par heure. Les protéines ont été éluées dans 300 ml d'un gradient linéaire de chlorure de sodium 0-250 mM dans du tampon A et des échantillons de 5 ml ont été recueillis. Les fractions contenant ULIP ont été collectées et du sulfate d'ammonium solide a été ajouté jusqu'à 20 % de saturation. Ce « pool » a été chargé sur une colonne CL-4B de phényl-Sepharose (1,75 cm<sup>2</sup> x 22 cm) qui a été préalablement équilibré avec 100 ml de tampon B (10 mM de phosphate de sodium, pH 7,8, 1 mM d'EGTA) contenant 20 % de sulfate d'ammonium saturé. Les protéines ont été éluées dans un gradient linéaire décroissant de 20 à 0 % de sulfate d'ammonium saturé dans du tampon B. Les fractions contenant ULIP ont été recueillies, dialysées deux fois contre 20 volumes de tampon A. Les protéines ont été concentrées sur une petite (10 ml) colonne C1-6B de DEAE-Sepharose et éluées avec 400 mM de chlorure de sodium dans du tampon A. L'éluat a été dessalé sur une colonne Sephadex G-25 (NAP-10) et concentré dans

un volume final de 0,5 ml par évaporation. Dans la dernière étape de purification, la fraction concentrée a été chromatographiée en trois étapes successives, sur deux colonnes de FPLC (Fast Protein Liquid Chromotagrophy) Superose 12 montées en série, dans du tampon C (50 mM de phosphate de sodium, pH 7,2, 150 mM de chlorure de sodium) à un débit de 0,3 ml/minute. Les fractions (0,6 ml) ont été recueillies et les fractions enrichies en ULIP ont été analysées. La présence d'ULIP dans les étapes successives de purification a été testée par un Western Blot à une dimension en utilisant un anticorps anti-stathmine capable de réactivité croisée. Les protéines ont été quantifiées selon la méthode de Bradford.

b) Migration sur gel d'électrophorèse :

Une électrophorèse à une dimension a été effectuée sur des gels de polyacrylamide 13 % selon la méthode de Laemmli. Les électrophorèses PAGE à deux dimensions ont été effectuées tel que décrit précédemment. Les gels d'isoélectrofocalisation contenaient 2 % d'ampholines total, pH 6-8 et 3-10 selon un rapport de 4 :1. La seconde dimension avait été menée sur des gels d'acrylamide à 10 %. Les protéines ont été soit soumises à une immuno-empreinte soit colorées par l'argent.

c) Analyse Western Blot :

Les protéines ont été transférées à partir des gels sur de la nitrocellulose dans du tampon contenant Tris 48 mM, glycine 39 mM et 5% de méthanol. La membrane a été saturée avec de la caséine (2,5 %) dans la solution d'immuno-empreinte (12 mM de Tris-HCl, pH 7,4, 160

- mM de Na Cl, 0,1 % de Triton X-100) et testée avec un antisérum dirigé contre le peptide I de la stathmine de rat (dilution 1/10.000) ou un antisérum dirigé contre la protéine ULIP recombinante (dilution 1/20.000) dilué dans une solution d'immuno-empreinte contenant 1 % de caséine.
- 5 Les anticorps liés ont été détectés soit avec une protéine A marquée à  $^{125}\text{I}$  et autoradiographiés soit avec des anticorps anti-lapin liés à la peroxydase en utilisant le kit ECL (Amersham).

d) Analyse de la séquence protéique :

- 10 Les fractions enrichies en ULIP ont été séparées sur les gels de polyacrylamide à deux dimensions. Les gels sont fixés pendant 30 minutes dans 25 % d'éthanol et 10 % d'acide acétique et colorés pendant 3 minutes dans 0,1 % de noir amido dans 1 % d'acide acétique et 40 % de méthanol. Les gels ont été décolorés dans 1 % d'acide acétique et les
- 15 taches correspondant à la forme principale de ULIP ont été découpées dans ces trois gels, recueillies et digérées avec 2 mg/ml d'endoprotéase Lys C. Les peptides élués du gel ont été ensuite séparés par HPLC sur colonne DEAE-C18 avec un gradient de 0-55 % d'acétonitrile dans 0,1 % d'acide trifluoroacétique. Les peptides ont ensuite été séquencés selon la
- 20 dégradation automatique d'Edman.

e) Expression *in vitro* chez un mammifère

- 1 µg du plasmide Bluescript contenant l'ADNc entier codant pour ULIP-1, ULIP-2, ULIP-3 ou ULIP-4 a été utilisé pour réaliser la
- 25 transcription et la traduction *in vitro* avec le système « Reticulocyte lysate » (Promega) selon le protocole décrit par le fabricant. 5 µg du

mélange de 25 µl total de transcription/traduction ont été analysés sur gel d'électrophorèse à deux dimensions.

### Résultats

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Ni la protéine recombinante ULIP-1, ni les protéines recombinantes TOAD-64 (ULIP-2) et C-22 (ULIP-3) ne sont reconnues par les sérums anti-CV2. De plus, le profil de distribution des taches correspondant à POP-66 reconnues par les anticorps anti-CV2 en électrophorèse à deux dimensions ne correspond pas aux taches reconnues par les anticorps anti-ULIP-1. Or, POP-66 est un membre de la famille ULIP puisque les trois taches POP-66 sont reconnues par l'Ac anti-pep3. POP-66 correspond donc à un membre de la famille de pHi plus basique.

15

Après traduction *in vitro* des quatre protéines (ULIP-1, 2, 3, 4), on a montré que ULIP-4 a le même profil électrophorétique 2D que POP-66 et est reconnue par les anticorps anti-CV2 (figure 7).

20

Pour cela, la protéine ULIP-4 et, comme contrôle, la protéine ULIP-2 ont été traduites *in vitro* en présence de méthionine <sup>35</sup>S à partir de clones d'ADNc codants pour les protéines entières. Les protéines ont été séparées par électrophorèse à deux dimensions (en présence d'un extrait de cerveau fournissant les repères essentiels), transférées sur nitrocellulose et révélées :

25

- par autoradiographie pour localiser les protéines traduites *in vitro* (traduction) ;
- par immunoempreinte avec le sérum CV2.



La figure 7 montre que les trois taches de la traduction *in vitro* d'ULIP-4 correspondent aux taches reconnues par CV2. Ces taches ne sont pas reconnues dans la traduction d'ULIP-2.

Le sérum CV2 reconnaît donc spécifiquement ULIP-4.

Ceci a permis d'identifier POP-66 comme ULIP-4.

#### **EXEMPLE 9 :**

##### **Localisation chromosomique de la protéine POP-66/ULIP-4**

L'ADNc d'ULIP-4 humain étant cloné, il est alors possible de déterminer la localisation chromosomique du gène POP-66/ULIP-4 par cartographie génique par hybridation *in situ* isotopique (Levy et Mattei et al., 1995).

L'hybridation *in situ* est menée sur des préparations de chromosomes obtenues à partir de lymphocytes humains stimulés par la phytohémagglutinine mis en culture pendant 72 heures. De la 5-bromodéoxyuridine a été ajoutée pendant les 7 dernières heures de la culture (60 µg/ml de milieu), pour assurer une image de bandes chromosomiques post-hybridation de bonne qualité. Le clone contenant un insert de 1300 paires de bases codant pour ULIP-4 dans le vecteur Bluescript est marqué au tritium par translation de coupure (« nick translation ») avec une activité spécifique de  $1 \times 10^8$  dpm. µg<sup>-1</sup>. La sonde radiomarquée a été hybridée au stade métaphase à une concentration finale de 200 ng par ml de solution d'hybridation. Après recouvrement par une émulsion Kodak NTB<sub>2</sub> les lames ont été exposées pendant 20 jours à +4°C puis développées. Pour éviter le décalage des grains d'argent

pendant le processus, les étalements de chromosomes ont été préalablement marqués avec une solution tampon de Giemsa et les métaphases ont été photographiées. La révélation des bandes a été effectuée par la méthode « fluorochrome-photolyse Giemsa » (FPG) et les

5 métaphases ont été rephotographiées avant analyse. Sur les 100 cellules en métaphase examinées après hybridation *in situ*, on a dénombré 246 grains d'argent associés aux chromosomes et 54 parmi ceux-ci (21.9 %) étaient localisés sur le chromosome 10. La distribution des grains sur ce chromosome n'était pas aléatoire : 39 sur 54 (72.2 % de ceux-ci) étaient

10 localisés sur la région q25.2-q26 du bras long du chromosome 10.

Le gène POP-66/ULIP-4 se trouve donc situé sur le chromosome 10 dans la région q25.2-q26. Dans cette région chromosomique, les locus de maladies neurodégénératives et de gènes suppresseurs de tumeurs impliqués dans différents types de cancers ont

15 été localisés. Le locus d'une maladie cérébelleuse à début précoce (« infantil onset spinocerebellar ataxia ») a été identifié dans la région 10q24-26 (Varilo et al., 1996 ; Nikali et al., 1995). Les symptômes de cette maladie dégénérative héréditaire récessive caractérisée par une ataxie, une neuropathie et une atrophie optique sont similaires à ceux

20 observés chez les patients atteints de syndromes neurologiques paranéoplasiques avec des auto-anticorps anti-CV2 circulants (Honnorat et al., 1996). D'un autre côté, 80 % des glioblastomes présentent des mutations dans cette région chromosomique et plusieurs locus suppressifs impliqués dans différents types de tumeurs (prostate, rein,

25 cancer du poumon à petites cellules et carcinomes de l'endomètre) sont localisés dans cette région chromosomique. Ces données renforcent la

possibilité que PO66/ULIP-4 joue un rôle crucial dans la neurodégénération et la tumorigénèse.

A cet égard, il est notable que l'expression de ULIP-1 est régulée à la hausse dans des cellules de neuroblastomes différenciés par l'acide rétinoïque et que ULIP-1 et ULIP-3 sont régulés à la hausse mais ULIP-4 est régulé à la baisse dans des cellules PC12 différenciées en présence de NGF, suggérant que l'arrêt de la croissance cellulaire peut être lié à des niveaux d'expression des protéines ULIP.

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#### **EXEMPLE 10 :**

#### **Expression des protéines ULIP dans les cellules HeLa transfectées**

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#### **A - Matériels et méthodes**

Une séquence « flag » (EcoRI-ATGGACTACAAGGACGACGATGACAAGG-BamHI) (Kodak) a été clonée dans le site EcoRI de pSG5 suivi par ULIP-1 (EMBEL X87817, paires de bases : 309-2023), Ulip2 (Y10339, paire de bases : 23-1741), Ulip3 (Y09080, paires de bases : 269-1991) ou Ulip 4 (Y09079, paires de bases : 102-1820), respectivement. Les cellules HeLa ont été cultivées dans des milieux DMEM (Gibco) additionnées de 10 % de sérum de veau foetal (v/v). Les transfections ont été réalisées par précipitation au phosphate de calcium (Maniatis et al., 1978). Les cellules HeLa ont été mélangées avec 5 µg de plasmides pSG5flag-ULIP-1, 2, 3, 4 et 10 µg de pUC18. Vingt quatre heures après la transfection, les cellules HeLa ont été fixées

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avec 4 % de paraformaldéhyde et immunomarquées avec différents sérums humains (dilution 1/300), révélées par des anticorps anti-IgG humains conjugués à la FITC (Biosys), ou des anticorps anti-flag (M2, Kodak) (dilution 1/1000), révélés par des anticorps anti-lapin conjugués au rouge Texas (Vector).

Un double immuno-marquage a été réalisé sur les cellules HeLa transfectées par des ULIP en utilisant des anticorps anti-flag et anti-Pep3. Dans les cellules transfectées par un quelconque ADNc, 10 à 20 % d'entre elles présentaient un immuno-marquage avec les anticorps anti-flag révélés par les anticorps anti-souris conjugués au rouge Texas.

Toutes les cellules transfectées ont été marquées en double par des anticorps dirigés contre Pep3, un peptide commun aux quatre ULIP est révélé par des anticorps anti-IgG de lapin conjugué à la fluoresceine.

Un double immuno-marquage a également été réalisé sur les cellules HeLa transfectées par des ULIP en utilisant des anticorps anti-flag et anti-CV2. Les sérums humains de patients atteints de SNP avec des auto-anticorps circulant anti-CV2 ont marqué les cellules transfectées par ULIP-4, et un sérum anti-CV2 a également marqué les cellules transfectées par ULIP-3. Aucun marquage des cellules transfectées par ULIP-4 n'a été détecté dans les sérums contrôles de patients sans cancer ou maladie neurologique.

## **B ) Résultats**

Après transfection des cellules HeLa avec des ADNc marqués par les flag des 4 ULIP, 10 à 20 % des cellules étaient fortement

réactives avec des anticorps anti-flag et des anticorps anti-Pep3 qui reconnaissent les 4 ULIP de mammifères. Les cellules transfectées n'ont pas été immuno-marquées avec du sérum contrôle de 10 patients neurologiques sans SNP ni avec du sérum pré-immun de lapin. En revanche, les cellules transfectées avec de l'ADNc d'ULIP-4 ont montré une immuno-réactivité intense avec tous les 7 sérums testés de patients avec des auto-anticorps anti-CV2 circulants. Ces sérums sont négatifs sur des cellules transfectées avec des ADNc d'autres ULIP, à l'exception d'un sérum qui a également reconnu les cellules transfectées par ULIP-3 et un sérum qui a également reconnu les cellules transfectées par ULIP-1, 3 et 4. Aucun marquage n'a été observé sur des cellules HeLa non transfectées, avec un sérum anti-CV2.

Le tableau I ci-après présente les résultats d'immunofluorescence indirecte avec différents sérums sur les cellules HeLa par des ADNc marqués de membres de la famille de ULIP.

Table 1 :

N° sérum	Symptômes neurologiques	Type de tumeur	Ulip-1	Ulip-2	Ulip-3	Ulip-4
Anti-Pep3	-	-	+	+	+	+
pre-immun	-	-	-	-	-	-
Pep3						
90-002	PCD, uvéite	UC	-	-	+	+
93-484	LE	Thymome	-	-	-	+
94-590	LE	SCLC	-	-	-	+
95-700	PEM	SCLC	+	-	+	+
95-701	PCD	sarcome utérin	-	-	-	+
95-706	LE, neuropathie	SCLC	-	-	-	+
97-040	PCD	SCLC	-	-	-	+
97-103	PCD	SCLC	-	-	-	+

- PCD : dégénération paranéoplasique du cervelet ;  
LE : encéphalite limbique ;  
PEM : encéphalomyélite paranéoplasique ;  
5 UC : carcinome indifférencié ;  
SCLC : carcinome de poumons à petites cellules.

### **EXEMPLE 11 :**

**Expression de POP-66/ULIP-4 et des membres de la**  
10 **famille ULIP dans les cancers**

#### ***A – Expression de ULIP-2 et ULIP-3 dans les cancers :***

##### **1) Matériels et méthodes : expériences de RT-PCR :**

15

L'ARN total a été extrait en utilisant 1 ml de RNAZOL™B (Bioprobe) selon la méthode de Chomczynski et Sacchi. La quantité d'ARN a été déterminée par densité optique mesurée à 260 nm et sa pureté a été déterminée à partir du rapport des absorbances mesurées à  
20 260 et 280 nm (rapports 1,8-2,0). L'intégrité des préparations d'ARN a été en outre vérifiée par électrophorèse sur gel d'agarose à 1 % dans du TBE (0,45 M de Tris-borate, 10 mM d'EDTA, pH 8). La spécificité des amorces a été analysée en comparant leurs séquences avec les diverses banques de données de gènes (EMBL et FASTA). Pour une quantification relative,  
25 le gène codant pour la G3PDH (glycéraldéhyde-3-phosphate déshydrogénase, Clontech), gène ubiquitaire exprimé dans de nombreux tissus incluant le cerveau, a été co-amplifié avec l'ARNm testé en tant que standard interne pour vérifier l'égalité des quantités d'ARN des échantillons et pour tester l'efficacité de l'étape de transcription inverse

pour les différents échantillons d'ARN. Les amorces 5', 3' et les oligonucléotides des sondes internes de G3PDH ont été synthétisées et purifiées par Eurogentec. L'ARNm total (1 µg) a été dénaturé (15 minutes à 65° C) et transcrit en ADNc simple brin (1 heure et demie, 42° C) dans  
5 un volume final de 20 µl de tampon (50 mM de Tris-HCl, 75 mM de KCl, pH 8,3, Gibco BRL) contenant 5 ng par µl d'amorce oligo-dT 12-18 (Pharmacia Biotech), 40 unités de reverse transcriptase du virus de la leucémie murine Moloney (Mu-LV) (Gibco BRL), 40 unités de RNAsine (Promega), 10 mM DTT (Gibco BRL) et 0,5 mM de chacun des  
10 déoxynucléotides triphosphate (Promega). Les échantillons d'ADNc ont été dilués au 1 /10 dans de l'eau distillée et les réaction de PCR ont été menées en utilisant 1 µl, 4 µl ou 2 µl de l'échantillon d'ADNc pour l'ARN messager d'ULIP-2 et ULIP-3, dans un tampon (50 mM de KCl, 10 mM de Tris-HCl, 0,1 % de Triton X100, 0,4 % de glycérol et 800 µM de NaCl, Ph  
15 9), dans lequel a été ajouté 40 µM de DTT, 3 mM de MgCl<sub>2</sub>, 0,2 mM de chaque dNTP, 0,4 µM de chaque amorce sélectionnée et 2 unités de l'AmpliTaq ADN polymérase (Promega) dans un volume final de 50 µl. Les échantillons ont ensuite été placés dans un thermocycleur (Biomed-Hybaid), dénaturés à 95°C pendant 5 minutes et amplifiés pendant 35  
20 cycles (un cycle = dénaturation 95° C pendant 65 secondes, hybridation des amorces 60° C pendant 45 secondes, extension 72° C pendant 4 minutes et allongement finale 15 minutes à 72° C. Les produits ont été séparés par électrophorèse sur gel d'agarose-Seakem à 1 % et les  
25 bandes tests des produits de RT-PCR de taille attendue ainsi que l'échelle de marqueur de poids moléculaire (100 paires de base) (Promega) ont été visualisés en utilisant la coloration au bromure d'éthidium.

Composition des sondes oligonucléotidiques utilisées pour la PCR ULIP-3  
5' ATAGAGGAGCGGATGACG (899) 3'

GCTGTTATGGTCTTCAACTTGTCGG (1092)

GGCCTGTTATGGTCTTCAACTTGTCG (1093)

5 Composition des sondes oligonucléotidiques utilisées pour la PCR ULIP-2  
5' AGGAGGAGTGAAGACCATCG 5227) 3'

CTTATGCCACTCGCTGATGTCC (509).

## 2) Résultats

10 Les expériences de RT-PCR montrent que TOAD-64  
(ULIP-2) et C-22 (ULIP-3) sont exprimés dans certaines tumeurs du  
poumon à petites cellules (cf. figure 8) et absents dans d'autres  
notamment dans celles des patients qui développent des syndromes  
neurologiques paranéoplasiques de meilleur pronostic.

15

### *B – Expression de ULIP-4 dans les cancers*

#### 1) Matériels et méthodes

20

##### • Préparation de l'ARN et RT-PCR

Les ARN totaux sont extraits de tumeurs cérébrales  
conservées dans l'azote liquide, selon la technique classique au  
RNAZOL™(Bioprobe, France). La transcription inverse a été effectuée en  
utilisant des oligo(dt)<sub>18</sub> sur 1 µg d'ARN total et la PCR a été réalisée avec  
25 1/20 du volume du mélange pour la transcription inverse (RT-mix). Les  
amorces utilisées pour ULIP-4 sont :

5'CATCTGGCTGTCGCTGCAC3', 5'GCCGCCCCTACCAGAGACC3',



et pour GAPDH : 5'GGAGATTCAGTGTGGTGG3',  
5'GGCTCTCCAGAACATCATCC3'. L'ADNc a été dénaturé à 95°C  
pendant cinq minutes. L'amplification par PCR a été réalisée pendant 30  
cycles. Ulip4 : 95°C, 45 sec ; 62°C, 45 sec ; 72°C, 45 sec. GAPDH : 95°C,  
5 45 sec ; 55°C, 45 sec ; 72°C, 45 sec. L'extension finale a été réalisée à  
72°C pendant 5 minutes.

## 2 ) Résultats

Sur les 8 extraits de glioblastomes étudiés, 4 (50 %)   
10 exprimaient l'ARN messenger de ULIP-4. A l'inverse, sur les 10 extraits  
d'oligodendrogliomes testés, aucun n'exprimait l'ARN messenger de ULIP-  
4. Cette expression différentielle, en fonction du type de tumeur cérébrale  
primitive, est en faveur d'un rôle potentiel de ULIP-4 dans la prolifération  
cellulaire de ces tumeurs.

15 La protéine POP-66/ULIP-4 ainsi que les protéines de la  
famille ULIP pourraient être exprimées dans les tumeurs périphériques  
(tumeur du poumon à petites cellules, thymome, cancer du sein et de  
l'ovaire). Leur présence pourrait donc être corrélée à un pronostic. La  
20 localisation du gène de POP-66/ULIP-4 sur la partie distale du  
chromosome 10 le confirme dans le cas des tumeurs cérébrales.

Ainsi, l'expression différentielle des membres de la famille  
ULIP dans des tumeurs telles que le cancer à petites cellules du poumon,  
25 alors que le gène ULIP correspondant est absent dans un tissu sain, ainsi  
que la modulation de l'expression des membres de la famille ULIP  
obtenus lors de la différenciation par le rétrovirus humain HTLV1 d'une

lignée de médulloblastome, suggèrent l'implication des ULIP dans les tumeurs cancéreuses.

### **EXEMPLE 12 :**

#### **5                    Production d'anticorps spécifiques de chacune des protéines ULIP humaines**

Des peptides spécifiques de chaque membre de la famille ULIP ont été synthétisés synthétisé sur un appareil de synthèse  
10    peptidique multiple utilisant le F-moc (432A Peptide Synthesizer SYNERGY, Applied Biosystems). La pureté a été vérifiée par analyse de la séquence par HPLC et spectrométrie de masse.

Ces peptides sont :

15    Peptide spécifique de ULIP-1 : G S A R G S P T R P N (11 acides aminés)

Peptide spécifique de ULIP-2 : S S A K T S P A K Q Q A (12 acides aminés)

20    Peptide spécifique de ULIP-3 : P S A K S S P S K H Q (11 acides aminés)

Peptide spécifique de ULIP-4 : P A R A S C P G K I S (11 acides aminés).

1 mg du peptide synthétique conjugué à de l'hémocyanine  
25    de patelle, dans de l'adjuvant complet de Freund, a été utilisé pour immuniser des lapins avec une dose « booster » de 0,5 mg de peptide lié dans de l'adjuvant complet de Freund après 4 semaines.

Les anticorps obtenus reconnaissent spécifiquement chaque protéine membre de la famille ULIP.

**EXEMPLE 13 :****Production d'animaux transgéniques exprimant ULIP-4**

Des drosophiles ont été transformées par l'ADNc d'ULIP-4  
5 humain.

L'ADNc de ULIP-4, précédemment cloné dans pbluescript SK-phagemid, a été excisé par double digestion enzymatique Kpn1 et Xba1. Après électrophorèse sur gel d'agarose, le fragment d'ADNc a été purifié puis cloné dans pUAST, dérivant de pCaSpeR3, digéré par les  
10 enzymes de restriction Kpn1 et Xba1. Le plasmide 10-C résulte du clonage directionnel de l'ADNc de ULIP-4 dans pUAST associé au gène rapporteur mini-white. Le plasmid 10-C a été injecté avec un plasmide helper p-delta-2-3 codant pour la transposase de l'élément P active dans la lignée germinale.

15 Les drosophiles transformées sont identifiées par leurs yeux rouges résultant de l'expression du gène mini-white. Ces lignées transformées par l'ADNc de ULIP-4 sous le contrôle de séquences régulatrices UASGAL4 permettent une expression ciblée de l'ADNc de ULIP-4.

20 Cette production de drosophiles transformées permet d'étudier spécifiquement le rôle de ULIP-4 dans différentes cellules et comprendre son implication dans les pathologies humaines.

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(ii) TITRE DE L'INVENTION: Utilisation des Ulip dans les SNP et les  
cancers associés

(iii) NOMBRE DE SEQUENCES: 8

## (iv) FORME LISIBLE PAR ORDINATEUR:

- (A) TYPE DE SUPPORT: Floppy disk
- (B) ORDINATEUR: IBM PC compatible
- (C) SYSTEME D' EXPLOITATION: PC-DOS/MS-DOS
- (D) LOGICIEL: PatentIn Release #1.0, Version #1.25 (OEB)

## (2) INFORMATION POUR LA SEQ ID NO: 1:

## (i) CARACTERISTIQUES DE LA SEQUENCE:

- (A) LONGUEUR: 1817 paires de bases
- (B) TYPE: acide nucléique
- (C) NOMBRE DE BRINS: simple
- (D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADNc

## (vi) ORIGINE:

- (A) ORGANISME: Mus musculus

## (xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 1:

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CAAAGTGATG CCCAAGAGTG CGGCTGAAGT CATCGCTCAG GCACGGAAGA AGGGAAGTGT 840  
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(2) INFORMATION POUR LA SEQ ID NO: 2:

## (i) CARACTERISTIQUES DE LA SEQUENCE:

- (A) LONGUEUR: 572 acides aminés
- (B) TYPE: acide aminé
- (C) NOMBRE DE BRINS: simple
- (D) CONFIGURATION: linéaire

## (ii) TYPE DE MOLECULE: peptide

## (vi) ORIGINE:

- (A) ORGANISME: Mus musculus

## (xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 2:

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Ala	Asp	Ile	Tyr	Met	Glu	Asp	Gly	Leu	Ile	Lys	Gln	Ile	Gly	Glu	Asn	35	40	45	
Leu	Ile	Val	Pro	Gly	Gly	Val	Lys	Thr	Ile	Glu	Ala	His	Ser	Arg	Met	50	55	60	
Val	Ile	Pro	Gly	Gly	Ile	Asp	Val	His	Thr	Arg	Phe	Gln	Met	Pro	Asp	65	70	75	80
Gln	Gly	Met	Thr	Ser	Ala	Asp	Asp	Phe	Phe	Gln	Gly	Thr	Lys	Ala	Ala	85	90	95	
Leu	Ala	Gly	Gly	Thr	Thr	Met	Ile	Ile	Asp	His	Val	Val	Pro	Glu	Pro	100	105	110	
Gly	Thr	Ser	Leu	Leu	Ala	Ala	Phe	Asp	Gln	Trp	Arg	Glu	Trp	Ala	Asp	115	120	125	
Ser	Lys	Ser	Cys	Cys	Asp	Tyr	Ser	Leu	His	Val	Asp	Ile	Thr	Glu	Trp	130	135	140	
His	Lys	Gly	Ile	Gln	Glu	Glu	Met	Glu	Ala	Leu	Val	Lys	Asp	His	Gly	145	150	155	160
Val	Asn	Ser	Phe	Leu	Val	Tyr	Met	Ala	Phe	Lys	Asp	Arg	Phe	Gln	Leu	165	170	175	
Thr	Asp	Ser	Gln	Ile	Tyr	Glu	Val	Leu	Ser	Val	Ile	Arg	Asp	Ile	Gly	180	185	190	
Ala	Ile	Ala	Gln	Val	His	Ala	Glu	Asn	Gly	Asp	Ile	Ile	Ala	Glu	Ala	195	200	205	



Gln Gln Arg Ile Leu Asp Leu Gly Ile Thr Gly Pro Glu Gly His Val  
 210 215 220  
 Leu Ser Arg Pro Glu Glu Val Glu Ala Glu Ala Val Asn Arg Ser Ile  
 225 230 235 240  
 Thr Ile Ala Asn Gln Thr Asn Cys Pro Leu Tyr Val Thr Lys Val Met  
 245 250 255  
 Pro Lys Ser Ala Ala Glu Val Ile Ala Gln Ala Arg Lys Lys Gly Thr  
 260 265 270  
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 His Tyr Trp Ser Lys Asn Trp Ala Lys Ala Ala Ala Phe Val Thr Ser  
 290 295 300  
 Pro Pro Leu Ser Pro Asp Pro Thr Thr Pro Asp Phe Leu Asn Ser Leu  
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 Leu Ser Cys Gly Asp Leu Gln Val Thr Gly Ser Ala His Cys Thr Phe  
 325 330 335  
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 340 345 350  
 Glu Gly Thr Asn Gly Thr Glu Glu Arg Met Ser Val Ile Trp Asp Lys  
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 Ala Val Val Thr Gly Lys Met Asp Glu Asn Gln Phe Val Ala Val Thr  
 370 375 380  
 Ser Thr Asn Ala Ala Lys Val Phe Asn Leu Tyr Pro Arg Lys Gly Arg  
 385 390 395 400  
 Ile Ser Val Gly Ser Asp Ala Asp Leu Val Ile Trp Asp Pro Asp Ser  
 405 410 415  
 Val Lys Thr Ile Ser Ala Lys Thr His Asn Ser Ala Leu Glu Tyr Asn  
 420 425 430  
 Ile Phe Glu Gly Met Glu Cys Arg Gly Ser Pro Leu Val Val Ile Ser  
 435 440 445  
 Gln Gly Lys Ile Val Leu Glu Asp Gly Thr Leu His Val Thr Glu Gly  
 450 455 460  
 Ser Gly Arg Tyr Ile Pro Arg Lys Pro Phe Pro Asp Phe Val Tyr Lys  
 465 470 475 480  
 Arg Ile Lys Ala Arg Ser Arg Leu Ala Glu Leu Arg Gly Val Pro Arg

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Val Thr Pro Ala Ser Ser Ala Lys Thr Ser Pro Ala Lys Gln Gln Ala	515		520		525
Pro Pro Val Arg Asn Leu His Gln Ser Gly Phe Ser Leu Ser Gly Ala	530		535		540
Gln Ile Asp Asp Asn Ile Pro Arg Arg Thr Thr Gln Arg Ile Val Ala	545		550		555
Pro Pro Gly Gly Arg Ala Asn Ile Thr Ser Leu Gly	565		570		

## (2) INFORMATION POUR LA SEQ ID NO: 3:

## (i) CARACTERISTIQUES DE LA SEQUENCE:

- (A) LONGUEUR: 2297 paires de bases
- (B) TYPE: acide nucléique
- (C) NOMBRE DE BRINS: simple
- (D) CONFIGURATION: linéaire

## (ii) TYPE DE MOLECULE: ADNc

## (vi) ORIGINE:

- (A) ORGANISME: Mus musculus

## (xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 3:

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TCTCACGAGT AGCGCCGCCG GGAGAGACCC GGGTAGAGCG CCAGGCAGAC GTTAGTTCCA	240
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AGATTGTCTT TGAGGATGGA AACATCAGTG TCAGCAAGGG CATGGGCCGC TTCATCCCTC 1680  
GGAAGCCATT CCCAGAGCAT CTCTACCAGC GTGTCAGGAT CAGAAGCAAG GTTTTCGGGT 1740  
TGCATAGTGT TTCCAGGGGC ATGTACGATG GGCCTGTGTA CGAGGTGCCA GCTACACCCA 1800  
AACATGCTGC TCCTGCTCCT TCTGCCGAAT CCTCGCCTTC TAAACACCAA CCCCCACCA 1860  
TCCGGAACCT CCACCAGTCC AACTTCAGCT TATCAGGTGC CCAGATAGAT GACAACAATC 1920  
CAAGGCGTAC AGGCCACCGC ATTGTGGCGC CCCCTGGTGG CCGCTCCAAC ATCACCAGCC 1980  
TCGGTTGACC TCAGATGAGC CAGATATGCA AGAGTGAAGG ATTATGGGAA AACGTCCATT 2040  
CCTTTTCCGT GTTTTTGAAG CCCACAGTTT TAGTTGGTAC TGACGGAGGG GAGATTGAGC 2100  
GATGCTCTTT CCTTCTCTGT TTAGGAAGAA GTGGTACTAG TGTGGTGTGT TTGCCTGGAA 2160  
GTCCCTCGCC CACAGTGTGT GTTCACACCG ACTCCACCTC AGAGCATGGT GCCGTCCGTT 2220

TTCCTTCCT AGTGACCCCA GGTTTAGCAT CGTCCTATAC TGTTCCTCC ACTCCTCCAT 2280  
 GACCCTCTGA GTGATGG 2297

(2) INFORMATION POUR LA SEQ ID NO: 4:

(i) CARACTERISTIQUES DE LA SEQUENCE:

- (A) LONGUEUR: 572 acides aminés
- (B) TYPE: acide aminé
- (C) NOMBRE DE BRINS: simple
- (D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: peptide

(vi) ORIGINE:

- (A) ORGANISME: Mus musculus

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 4:

Met	Ser	His	Gln	Gly	Lys	Lys	Ser	Ile	Pro	His	Ile	Thr	Ser	Asp	Arg	1	5	10	15
Leu	Leu	Ile	Arg	Gly	Gly	Arg	Ile	Ile	Asn	Asp	Asp	Gln	Ser	Phe	Tyr	20	25	30	
Ala	Asp	Val	Tyr	Leu	Glu	Asp	Gly	Leu	Ile	Lys	Gln	Ile	Gly	Glu	Asn	35	40	45	
Leu	Ile	Val	Pro	Gly	Gly	Val	Lys	Thr	Ile	Glu	Ala	Asn	Gly	Arg	Met	50	55	60	
Val	Ile	Pro	Gly	Gly	Ile	Asp	Val	Asn	Thr	Tyr	Leu	Gln	Lys	Pro	Ser	65	70	75	80
Gln	Gly	Met	Thr	Ser	Ala	Asp	Asp	Phe	Phe	Gln	Gly	Thr	Lys	Ala	Ala	85	90	95	
Leu	Ala	Gly	Gly	Thr	Thr	Met	Ile	Ile	Asp	His	Val	Val	Pro	Glu	Pro	100	105	110	
Gly	Ser	Ser	Leu	Leu	Thr	Ser	Phe	Glu	Lys	Trp	His	Glu	Ala	Ala	Asp	115	120	125	
Thr	Lys	Ser	Cys	Cys	Asp	Tyr	Ser	Leu	His	Val	Asp	Ile	Thr	Ser	Trp	130	135	140	
Tyr	Asp	Gly	Val	Arg	Glu	Glu	Leu	Glu	Val	Leu	Val	Gln	Asp	Lys	Gly	145	150	155	160
Val	Asn	Ser	Phe	Gln	Val	Tyr	Met	Ala	Tyr	Lys	Asp	Leu	Tyr	Gln	Met	165	170	175	

Ser Asp Ser Gln Leu Tyr Glu Ala Phe Thr Phe Leu Lys Gly Leu Gly  
 180 185 190  
 Ala Val Ile Leu Val His Ala Glu Asn Gly Asp Leu Ile Ala Gln Glu  
 195 200 205  
 Gln Lys Arg Ile Leu Glu Met Gly Ile Thr Gly Pro Glu Gly His Ala  
 210 215 220  
 Leu Ser Arg Pro Glu Glu Leu Glu Ala Glu Ala Val Phe Arg Ala Ile  
 225 230 235 240  
 Ala Ile Ala Gly Arg Ile Asn Cys Pro Val Tyr Ile Thr Lys Val Met  
 245 250 255  
 Ser Lys Ser Ala Ala Asp Ile Ile Ala Leu Ala Arg Lys Lys Gly Pro  
 260 265 270  
 Leu Val Phe Gly Glu Pro Ile Ala Ala Ser Leu Gly Thr Asp Gly Thr  
 275 280 285  
 His Tyr Trp Ser Lys Asn Trp Ala Lys Ala Ala Ala Phe Val Thr Ser  
 290 295 300  
 Pro Pro Leu Ser Pro Asp Pro Thr Thr Pro Asp Tyr Leu Thr Ser Leu  
 305 310 315 320  
 Leu Ala Cys Gly Asp Leu Gln Val Thr Gly Ser Gly His Cys Pro Tyr  
 325 330 335  
 Ser Ile Ala Gln Lys Ala Val Gly Lys Asp Asn Phe Thr Leu Ile Pro  
 340 345 350  
 Glu Gly Val Asn Gly Ile Glu Glu Arg Met Thr Val Val Trp Asp Lys  
 355 360 365  
 Ala Val Ala Thr Gly Lys Met Asp Glu Asn Gln Phe Val Ala Val Thr  
 370 375 380  
 Ser Thr Asn Ala Ala Lys Ile Phe Asn Leu Tyr Pro Arg Lys Gly Arg  
 385 390 395 400  
 Ile Ala Val Gly Ser Asp Ala Asp Val Val Ile Trp Asp Pro Asp Lys  
 405 410 415  
 Met Lys Thr Ile Thr Ala Lys Ser His Lys Ser Thr Val Glu Tyr Asn  
 420 425 430  
 Ile Phe Glu Gly Met Glu Cys His Gly Ser Pro Leu Val Val Ile Ser  
 435 440 445  
 Gln Gly Lys Ile Val Phe Glu Asp Gly Asn Ile Ser Val Ser Lys Gly

450		455		460
Met Gly Arg Phe Ile Pro Arg Lys Pro Phe Pro Glu His Leu Tyr Gln				
465		470		475
Arg Val Arg Ile Arg Ser Lys Val Phe Gly Leu His Ser Val Ser Arg				
	485		490	495
Gly Met Tyr Asp Gly Pro Val Tyr Glu Val Pro Ala Thr Pro Lys His				
	500		505	510
Ala Ala Pro Ala Pro Ser Ala Glu Ser Ser Pro Ser Lys His Gln Pro				
	515		520	525
Pro Pro Ile Arg Asn Leu His Gln Ser Asn Phe Ser Leu Ser Gly Ala				
	530		535	540
Gln Ile Asp Asp Asn Asn Pro Arg Arg Thr Gly His Arg Ile Val Ala				
	545		550	555
Pro Pro Gly Gly Arg Ser Asn Ile Thr Ser Leu Gly				
	565		570	

## (2) INFORMATION POUR LA SEQ ID NO: 5:

## (i) CARACTERISTIQUES DE LA SEQUENCE:

- (A) LONGUEUR: 1920 paires de bases
- (B) TYPE: acide nucléique
- (C) NOMBRE DE BRINS: simple
- (D) CONFIGURATION: linéaire

## (ii) TYPE DE MOLECULE: ADNc

## (vi) ORIGINE:

- (A) ORGANISME: Mus musculus

## (xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 5:

GCTGACTAAT ATGCTTAAAT TCAGCGGGTC GCCACGTCTG GTCGGTACGT CCACGCCCCG	60
GCAGCCCCTA CCGAGGACAC TCAGCCCGCC CGTGTATCAG GATGTCCTTC CAAGGCAAGA	120
AGAGCATTCC CCGGATAACG AGCGACCGCC TTCTCATCAA AGGTGGGAAG ATTGTGAACG	180
ATGACCAGTC CTTTCATGCT GATCTGTATG TGGAAGACGG TCTGATTAAA CAAATTGGAG	240
AAAATCTCAT CGTCCCTGGG GGCATCAAAA CCATCGATGC TCATGGCCTG ATGGTGCTGC	300
CTGGGGGAGT TGACGTTTAC ACCCGGCTGC AGATGCCTGT GATGGGCATG ACCCCAGCTG	360
ATGATTTCTG TCAGGGCACC AAGGCGGCTC TAGCAGGCGG GACCACCATG ATATTGGACC	420

ATGTGTTTCC TGACGCTGGT GTGAGCCTGC TGGCAGCCTA TGAGCAGTGG CGGGACGGAG	480
CAGACAGCGC GGCCTGCTGT GACTACTCCT TACATGTGGA CATTCCTCGC TGGCACGAGA	540
GCACCAAAGA AGAGCTGGAG GCCCTAGTCA GGGACAAAGG TGTGAACTCC TTCCTGGTCT	600
TCATGGCATA CAAGGACAGG TGCCAGTGTA CTGACGGTCA GATATATGAA ATCTTCAGCC	660
TCATCCGGGA CCTGGGAGCT GTGGCCAGG TGCACGCAGA AAATGGGGAC ATCGTGGAGG	720
AGGAACAGAA GCGCCTGCTG GAGCAAGGCA TCACTGGTCC TGAGGGCCAT GTGCTCAGCC	780
ACCCAGAAGA GGTAGAGGCC GAGGCTGTGT ACAGAGCAGT CACCATTGCC AAGCAGGCCA	840
ACTGCCCCACT ATACGTCACC AAGGTGATGA GCAAGGGTGC AGCTGACATG GTTGCCCAAG	900
CCAAGCGCAG GGGGGTGGTC GTCTTTGGGG AACCTATCAC TGCCAGCCTG GGCCTGATG	960
GCTCACACTA CTGGAGCAAG AACTGGGCCA AGGCTGCAGC CTTTGTCACT TCACCCCTA	1020
TCAACCCGGA CCCTACTACT GCAGACCACC TCACCTCTCT GCTGTCCAGT GGGGACCTCC	1080
AGGTGACAGG CAGTGCCAC TGCACCTTCA CTA CTGCCCCA GAAGGCTGTT GGCAAAGACA	1140
ACTTCACACT GATCCCCGAG GTAGTCAACG GTATAGAAGA GCGCATGTCT GTGGTCTGGG	1200
AGAAATGTGT GGCTTCAGGG AAAATGGACG AGAATGAGTT CGTTGCCGTG ACCAGCACAA	1260
ATGCTGCCAA AATCTTCAAT TTTTACCCCA GGAAGGGGCG TGTGGCCGTG GGCTCTGATG	1320
CTGACCTGGT CATCTGGAAC CCCAGGGCCA CGAAAGTCAT CTCTGCCAAG AGCCATAACC	1380
TGAATGTAGA GTACAACATC TTTGAAGGAG TGGAGTGCCG AGGAGTGCCC ACGGTGGTCA	1440
TAAGTCAGGG CAGAGTGGTG CTGGAGGACG GAAACCTGCT TGTCCTCCA GGGGCTGGCC	1500
GCTTCATTCC CCGGAAGACG TTCCCGGACT TTGTCTATAA GAGGATAAAG GCTCGCAACA	1560
GGCTAGCAGA GATCCACGGT GTGCCTCGAG GCCTGTACGA CGGGCCTGTG CATGAAGTGA	1620
TGTTACCTGC CAAGCCAGGA AGTGGCACAC AGGCCCGTGC ATCCTGTTCA GGCAAGATCT	1680
CAGTGCCACC CGTGCGCAAC CTGCACCAGT CGGGGTTCAG CCTATCTGGC TCTCAGGCTG	1740
ACGATCACAT TGCCAGACGT ACGGCTCAGA AGATCATGGC ACCCCCCGGA GGACGCTCCA	1800
ACATCACGTC TCTTTCCTAG ACTTGGGGTC TTGGCAAGCT GGTGCTGTCC CCACTGGCAG	1860
GGTGTGGGGA CGACTCACGT CAGTTAGCTC CTTCTTTGT AGATTGTTAT TGTGAAAGGC	1920

(2) INFORMATION POUR LA SEQ ID NO 6:

## (i) CARACTERISTIQUES DE LA SEQUENCE:

- (A) LONGUEUR: 572 acides aminés
- (B) TYPE: acide aminé
- (C) NOMBRE DE BRINS: simple
- (D) CONFIGURATION: linéaire

## (ii) TYPE DE MOLECULE: peptide

## (vi) ORIGINE:

- (A) ORGANISME: Mus musculus

## (xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO 6:

Met	Ser	Phe	Gln	Gly	Lys	Lys	Ser	Ile	Pro	Arg	Ile	Thr	Ser	Asp	Arg	1	5	10	15
Leu	Leu	Ile	Lys	Gly	Gly	Lys	Ile	Val	Asn	Asp	Asp	Gln	Ser	Phe	His	20	25	30	
Ala	Asp	Leu	Tyr	Val	Glu	Asp	Gly	Leu	Ile	Lys	Gln	Ile	Gly	Glu	Asn	35	40	45	
Leu	Ile	Val	Pro	Gly	Gly	Ile	Lys	Thr	Ile	Asp	Ala	His	Gly	Leu	Met	50	55	60	
Val	Leu	Pro	Gly	Gly	Val	Asp	Val	His	Thr	Arg	Leu	Gln	Met	Pro	Val	65	70	75	80
Met	Gly	Met	Thr	Pro	Ala	Asp	Asp	Phe	Cys	Gln	Gly	Thr	Lys	Ala	Ala	85	90	95	
Leu	Ala	Gly	Gly	Thr	Thr	Met	Ile	Leu	Asp	His	Val	Phe	Pro	Asp	Ala	100	105	110	
Gly	Val	Ser	Leu	Leu	Ala	Ala	Tyr	Glu	Gln	Trp	Arg	Asp	Gly	Ala	Asp	115	120	125	
Ser	Ala	Ala	Cys	Cys	Asp	Tyr	Ser	Leu	His	Val	Asp	Ile	Pro	Arg	Trp	130	135	140	
His	Glu	Ser	Thr	Lys	Glu	Glu	Leu	Glu	Ala	Leu	Val	Arg	Asp	Lys	Gly	145	150	155	160
Val	Asn	Ser	Phe	Leu	Val	Phe	Met	Ala	Tyr	Lys	Asp	Arg	Cys	Gln	Cys	165	170	175	
Thr	Asp	Gly	Gln	Ile	Tyr	Glu	Ile	Phe	Ser	Leu	Ile	Arg	Asp	Leu	Gly	180	185	190	
Ala	Val	Ala	Gln	Val	His	Ala	Glu	Asn	Gly	Asp	Ile	Val	Glu	Glu	Glu	195	200	205	



Gln Lys Arg Leu Leu Glu Gln Gly Ile Thr Gly Pro Glu Gly His Val  
 210 215 220  
 Leu Ser His Pro Glu Glu Val Glu Ala Glu Ala Val Tyr Arg Ala Val  
 225 230 235 240  
 Thr Ile Ala Lys Gln Ala Asn Cys Pro Leu Tyr Val Thr Lys Val Met  
 245 250 255  
 Ser Lys Gly Ala Ala Asp Met Val Ala Gln Ala Lys Arg Arg Gly Val  
 260 265 270  
 Val Val Phe Gly Glu Pro Ile Thr Ala Ser Leu Gly Thr Asp Gly Ser  
 275 280 285  
 His Tyr Trp Ser Lys Asn Trp Ala Lys Ala Ala Ala Phe Val Thr Ser  
 290 295 300  
 Pro Pro Ile Asn Pro Asp Pro Thr Thr Ala Asp His Leu Thr Ser Leu  
 305 310 315 320  
 Leu Ser Ser Gly Asp Leu Gln Val Thr Gly Ser Ala His Cys Thr Phe  
 325 330 335  
 Thr Thr Ala Gln Lys Ala Val Gly Lys Asp Asn Phe Thr Leu Ile Pro  
 340 345 350  
 Glu Val Val Asn Gly Ile Glu Glu Arg Met Ser Val Val Trp Glu Lys  
 355 360 365  
 Cys Val Ala Ser Gly Lys Met Asp Glu Asn Glu Phe Val Ala Val Thr  
 370 375 380  
 Ser Thr Asn Ala Ala Lys Ile Phe Asn Phe Tyr Pro Arg Lys Gly Arg  
 385 390 395 400  
 Val Ala Val Gly Ser Asp Ala Asp Leu Val Ile Trp Asn Pro Arg Ala  
 405 410 415  
 Thr Lys Val Ile Ser Ala Lys Ser His Asn Leu Asn Val Glu Tyr Asn  
 420 425 430  
 Ile Phe Glu Gly Val Glu Cys Arg Gly Val Pro Thr Val Val Ile Ser  
 435 440 445  
 Gln Gly Arg Val Val Leu Glu Asp Gly Asn Leu Leu Val Thr Pro Gly  
 450 455 460  
 Ala Gly Arg Phe Ile Pro Arg Lys Thr Phe Pro Asp Phe Val Tyr Lys  
 465 470 475 480  
 Arg Ile Lys Ala Arg Asn Arg Leu Ala Glu Ile His Gly Val Pro Arg  
 485 490 495

Gly Leu Tyr Asp Gly Pro Val His Glu Val Met Leu Pro Ala Lys Pro  
                   500                                  505                                  510

Gly Ser Gly Thr Gln Ala Arg Ala Ser Cys Ser Gly Lys Ile Ser Val  
                   515                                  520                                  525

Pro Pro Val Arg Asn Leu His Gln Ser Gly Phe Ser Leu Ser Gly Ser  
                   530                                  535                                  540

Gln Ala Asp Asp His Ile Ala Arg Arg Thr Ala Gln Lys Ile Met Ala  
                   545                                  550                                  555                                  560

Pro Pro Gly Gly Arg Ser Asn Ile Thr Ser Leu Ser  
                                   565                                  570

## (2) INFORMATION POUR LA SEQ ID NO 7 :

## (i) CARACTERISTIQUES DE LA SEQUENCE:

- (A) LONGUEUR: 1690 paires de bases
- (B) TYPE: acide nucléique
- (C) NOMBRE DE BRINS: simple
- (D) CONFIGURATION: linéaire

## (ii) TYPE DE MOLECULE: ADNc

## (vi) ORIGINE:

- (A) ORGANISME: Homo sapiens

## (xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO 7:

GCCGCCCCCTA CCAGAGACCC CCAGGAGCAG GATGTCCTTC CAGGGCAAGA AAAGCATCCC	60
CCGGATCACG AGTGACCGCC TTCTGATCAG AGGTGGGAGG ATCGTGAATG ACGACCAGTC	120
CTTTTACGCT GATGTGCACG TGGAAGATGG CTTGATAAAA CAAATCGGAG AAAACCTCAT	180
CGTCCCTGGG GGCATCAAGA CCATTGACGC CCACGGCCTG ATGGTCCTTC CTGGTGGCGT	240
TGACGTCCAC ACAAGGCTGC AGATGCCTGT CCTGGGCATG ACACCGGCTG ACGACTTCTG	300
TCAGGGCACC AAGGCAGCGC TAGCAGGAGG AACCACCATG ATCTTGGACC ACGTCTTCCC	360
CGACACGGGT GTGAGCCTGC TGGCGGCCTA CGAGCAGTGG CGGGAGCGGG CGGACAGCGC	420
GGCCTGCTGC GACTACTCCC TGCACGTGGA CATCACCCGA TGGCATGAGA GCATCAAGGA	480
GGAGCTGGAG GCCCTGGTCA AGGAGAAGGG TGTGAACTCC TTCCTGGTCT TCATGGCATA	540
CAAGGACCGG TGCCAGTGCA GCGACAGCCA GATGTACGAG ATCTTCAGCA TCATCCGGGA	600
CCTGGGGGCC TTGGCCCAGG TGCACGCTGA GAACGGGGAC ATCGTGGAGG AGGAGCAGAA	660

GCGGTTGCTG GAGCTCGGCA TCACTGGCCC CGAGGGCCAC GTGCTCAGCC ACCCCGAGGA 720  
GGTGGAGGCT GAGGCGGTGT ACCGAGCTGT CACCATCGCC AAGCAGGCAA ACTGCCCCGCT 780  
GTACGTCACC AAGGTGATGA GCAAGGGGGC GGCCGACGCC ATCGCTCAGG CCAAGCGCAG 840  
AGGGGTGGTC GTGTTTGGGG AGCCCATCAC CGCCAGCCTG GGCACCGACG GTTCACACTA 900  
CTGGAGCAAG AACTGGGCCA AGGCTGCAGC CTTGTCACA TCACCCCTG TCAACCCAGA 960  
CCCCACCACG GCAGACCACC TCACCTGCTT GCTGTCCAGC GGGGACCTCC AGGTGACAGG 1020  
CAGCGCCCAC TGCACCTTCA CCACTGCCCCA GAAGGCTGTG GGCAAGGACA ACTTCGCGCT 1080  
GATCCCCGAG GGCACCAACG GCATTGAGGA GCGCATGTCG ATGGTCTGGG AGAAATGTGT 1140  
GGCCTCTGGG AAGATGGACG AGAATGAGTT CGTCGCGGTG ACCAGTACAA ATGCTGCCAA 1200  
AATCTTCAAT TTTTACCCAA GGAAGGGGCG AGTGGCTGTG GGCTCTGACG CTGACCTGGT 1260  
CATATGGAAC CCAAGGCCA CCAAGATCAT CTCTGCCAAG ACCCACAATC TGAACGTGGA 1320  
GTACAACATC TTCGAGGGAG TGGAGTGCCG GGGAGCGCCT GCCGTGGTCA TAAGTCAGGG 1380  
CCGAGTGCGG CTGGAGGACG GGAAGATGTT TGTCACCCCG GGGGCGGGCC GCTTCGTCCC 1440  
TCGGAAAACA TTCCCGGACT TTGTCTACAA GAGGATCAAA GCTCGCAACA GGCTGGCGGA 1500  
GATCCACGGT GTGCCCCGTG GGCTGTATGA CGGGCCCGTC CACGAGGTGA TGGTGCCTGC 1560  
CAAGCCAGGG AGTGGCGCTC CGGCCCCGCGC GTCCTGCCCA GGCAAGATCT CCGTGCCTCC 1620  
TGTGCGCAAC CTACATCAGT CGGGGTTTCTAG CCTATCTGGG TCTCAGGCTG ATGACCACAT 1680  
CGCCCGACGC 1690

(2) INFORMATION POUR LA SEQ ID NO 8 :

(i) CARACTERISTIQUES DE LA SEQUENCE:

- (A) LONGUEUR: 572 acides aminés
- (B) TYPE: acide aminé
- (C) NOMBRE DE BRINS: simple
- (D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: peptide

(vi) ORIGINE:

- (A) ORGANISME: Homo sapiens

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO 8 :

Met Ser Phe Gln Gly Lys Lys Ser Ile Pro Arg Ile Thr Ser Asp Arg  
 1 5 10 15  
 Leu Leu Ile Arg Gly Gly Arg Ile Val Asn Asp Asp Gln Ser Phe Tyr  
 20 25 30  
 Ala Asp Val His Val Glu Asp Gly Leu Ile Lys Gln Ile Gly Glu Asn  
 35 40 45  
 Leu Ile Val Pro Gly Gly Ile His Thr Ile Asp Ala His Gly Leu Met  
 50 55 60  
 Val Leu Pro Gly Gly Val Asp Val His Thr Arg Leu Gln Met Pro Val  
 65 70 75 80  
 Leu Gly Met Thr Pro Ala Asp Asp Phe Cys Gln Gly Thr Lys Ala Ala  
 85 90 95  
 Leu Ala Gly Gly Thr Thr Met Ile Leu Asp His Val Phe Pro Asp Thr  
 100 105 110  
 Gly Val Ser Leu Leu Ala Ala Tyr Glu Gln Trp Arg Glu Arg Ala Asp  
 115 120 125  
 Ser Ala Ala Cys Cys Asp Tyr Ser Leu His Val Asp Ile Thr Arg Trp  
 130 135 140  
 His Glu Ser Ile Lys Glu Glu Leu Glu Ala Leu Val Lys Glu Lys Gly  
 145 150 155 160  
 Val Asn Ser Phe Leu Val Phe Met Ala Tyr Lys Asp Arg Cys Gln Cys  
 165 170 175  
 Ser Asp Ser Gln Met Tyr Glu Ile Phe Ser Ile Ile Arg Asp Leu Gly  
 180 185 190  
 Ala Leu Ala Gln Val His Ala Glu Asn Gly Asp Ile Val Glu Glu Glu  
 195 200 205  
 Gln Lys Arg Leu Leu Glu Leu Gly Ile Thr Gly Pro Glu Gly His Val  
 210 215 220  
 Leu Ser His Pro Glu Glu Val Glu Ala Glu Ala Val Tyr Arg Ala Val  
 225 230 235 240  
 Thr Ile Ala Lys Gln Ala Asn Cys Pro Leu Tyr Val Thr Lys Val Met  
 245 250 255  
 Ser Lys Gly Ala Ala Asp Ala Ile Ala Gln Ala Lys Arg Arg Gly Val  
 260 265 270  
 Val Val Phe Gly Glu Pro Ile Thr Ala Ser Leu Gly Thr Asp Gly Ser  
 275 280 285

His Tyr Trp Ser Lys Asn Trp Ala Lys Ala Ala Ala Phe Val Thr Ser  
 290 295 300  
 Pro Pro Val Asn Pro Asp Pro Thr Thr Ala Asp His Leu Thr Cys Leu  
 305 310 315 320  
 Leu Ser Ser Gly Asp Leu Gln Val Thr Gly Ser Ala His Cys Thr Phe  
 325 330 335  
 Thr Thr Ala Gln Lys Ala Val Gly Lys Asp Asn Phe Ala Leu Ile Pro  
 340 345 350  
 Glu Gly Thr Asn Gly Ile Glu Glu Arg Met Ser Met Val Trp Glu Lys  
 355 360 365  
 Cys Val Ala Ser Gly Lys Met Asp Glu Asn Glu Phe Val Ala Val Thr  
 370 375 380  
 Ser Thr Asn Ala Ala Lys Ile Phe Asn Phe Tyr Pro Arg Lys Gly Arg  
 385 390 395 400  
 Val Ala Val Gly Ser Asp Ala Asp Leu Val Ile Trp Asn Pro Lys Ala  
 405 410 415  
 Thr Lys Ile Ile Ser Ala Lys Thr His Asn Leu Asn Val Glu Tyr Asn  
 420 425 430  
 Ile Phe Glu Gly Val Glu Cys Arg Gly Ala Pro Ala Val Val Ile Ser  
 435 440 445  
 Gln Gly Arg Val Ala Leu Glu Asp Gly Lys Met Phe Val Thr Pro Gly  
 450 455 460  
 Ala Gly Arg Phe Val Pro Arg Lys Thr Phe Pro Asp Phe Val Tyr Lys  
 465 470 475 480  
 Arg Ile Lys Ala Arg Asn Arg Leu Ala Glu Ile His Gly Val Pro Arg  
 485 490 495  
 Gly Leu Tyr Asp Gly Pro Val His Glu Val Met Val Pro Ala Lys Pro  
 500 505 510  
 Gly Ser Gly Ala Pro Ala Arg Ala Ser Cys Pro Gly Lys Ile Ser Val  
 515 520 525  
 Pro Pro Val Arg Asn Leu His Gln Ser Gly Phe Ser Leu Ser Gly Ser  
 530 535 540  
 Gln Ala Asp Asp His Ile Ala Arg Arg Thr Ala Gln Lys Ile Met Ala  
 545 550 555 560  
 Pro Pro Gly Gly Arg Ser Asn Ile Thr Ser Leu Ser  
 565 570

## REVENDICATIONS

1. Polypeptide purifié, dérivé ou fragment polypeptidique dudit polypeptide purifié biologiquement actif, comprenant une séquence  
5 d'acides aminés choisie parmi SEQ ID n° 2, n° 4, n° 6 et n° 8.

2. Polypeptide purifié, dérivé ou fragment polypeptidique biologiquement actif dudit polypeptide purifié, selon la revendication 1 comprenant la séquence d'acides aminés SEQ ID n° 8, ledit polypeptide étant désigné par « POP-66 ».

10 3. Séquence nucléotidique isolée, comprenant :

- une séquence choisie parmi SEQ ID n° 1, n° 3, n° 5 et n° 7 codant pour un polypeptide de séquence d'acides aminés respectivement SEQ ID n° 2, n° 4, n° 6 et n° 8 ;

- une séquence dérivée d'une séquence choisie parmi  
15 SEQ ID n° 1, n° 3, n° 5 et n° 7 du fait de la dégénérescence du code génétique, de mutation, de délétion ou d'insertion ;

- ou une séquence capable de s'hybrider spécifiquement avec la séquence SEQ ID N° 1, n° 3, n° 5 ou n° 7.

20 4. Séquence nucléotidique selon la revendication 3, comprenant la séquence nucléique SEQ ID n° 7 codant pour un polypeptide selon la revendication 2.

5. Vecteur de clonage et/ou d'expression contenant une séquence d'acides nucléiques selon l'une des revendications 3 et 4.

25 6. Cellule hôte transfectée par un vecteur selon la revendication 5.

7. Anticorps mono- ou polyclonaux obtenus à partir d'un polypeptide selon l'une des revendications 1 et 2, purifié, dérivé ou

fragment polypeptidique biologiquement actif dudit polypeptide purifié, ainsi que les fragments, les anticorps chimériques ou les immunoconjugués desdits anticorps mono- ou polyclonaux.

8. Composition utile pour le diagnostic des syndromes  
5 neurologiques paranéoplasiques et/ou pour le diagnostic précoce de la formation des tumeurs, caractérisée en ce qu'elle comprend un polypeptide purifié POP-66, dérivé ou fragment polypeptidique biologiquement actif de POP-66 selon la revendication 2.

9. Utilisation d'un polypeptide purifié POP 66, dérivé ou  
10 fragment polypeptidique biologiquement actif de POP-66 selon la revendication 2 ou d'une séquence nucléotidique selon la revendication 4 pour détecter la présence d'anticorps anti-CV2 dans un échantillon biologique.

10. Utilisation d'anticorps mono- ou polyclonaux ou leurs  
15 fragments, anticorps chimériques ou immunoconjugués selon la revendication 7, pour la purification ou la détection d'une protéine ULIP correspondante dans un échantillon biologique.

11. Utilisation d'anticorps dirigés contre une protéine de la  
famille ULIP pour la mise en évidence d'une protéine ULIP dans des  
20 néoplasmes et les syndromes neurologiques paranéoplasiques, à des fins de diagnostic.

12. Utilisation selon la revendication 11, les anticorps étant  
des anticorps monoclonaux obtenus à partir du sérum polyclonal anti-CV2 de patients.

25 13. Méthode pour le diagnostic des syndromes neurologiques paranéoplasiques et/ou pour le diagnostic précoce de la formation des tumeurs cancéreuses, caractérisée en ce que l'on met en

évidence dans un échantillon de sang prélevé chez un individu des auto-anticorps dirigés contre une protéine POP-66 par

- la mise en contact un échantillon de sang prélevé chez un individu avec un polypeptide purifié (POP-66), dérivé ou fragment polypeptidique biologiquement actif de POP-66 selon la revendication 2, éventuellement fixé sur un support dans des conditions permettant la formation de complexes immunologiques spécifiques entre ledit polypeptide et les auto-anticorps éventuellement présents dans l'échantillon de sang, et
- la détection des complexes immunologiques spécifiques éventuellement formés.

14. Kit pour le diagnostic des syndromes neurologiques paranéoplasiques et pour le diagnostic précoce de la formation des tumeurs à partir d'un prélèvement biologique comprenant :

- au moins un polypeptide purifié POP-66, dérivé ou fragment polypeptidique biologiquement actif de POP-66, selon la revendication 2 éventuellement fixé sur un support,
- des moyens de révélation de la formation de complexes antigène/anticorps spécifiques entre un auto-anticorps anti-POP-66 et ledit polypeptide purifié POP-66, dérivé ou fragment polypeptidique et/ou des moyens de quantification de ces complexes.

15. Composition pharmaceutique, comprenant au moins une protéine purifiée de la famille ULIP, fragment polypeptidique ou dérivé biologiquement actif de celle-ci, une séquence ou fragment de séquence nucléotidique codant pour ladite protéine, une séquence anti-sens capable de s'hybrider spécifiquement avec une séquence nucléotidique



codant pour ladite protéine, ou un anticorps dirigé contre ladite protéine, associée à un véhicule pharmaceutiquement acceptable.

16. Composition pharmaceutique selon la revendication 15, comprenant au moins un polypeptide purifié POP-66 selon la revendication 2, fragment polypeptidique ou dérivé biologiquement actif de celui-ci, une séquence ou fragment de séquence nucléotidique codant pour ledit polypeptide, une séquence anti-sens capable de s'hybrider spécifiquement avec une séquence nucléotidique codant pour ledit polypeptide, ou un anticorps dirigé contre ledit polypeptide, associé à un véhicule pharmaceutiquement acceptable.

17. Utilisation d'une protéine purifiée de la famille ULIP, fragment polypeptidique ou dérivé biologiquement actif de celle-ci, une séquence ou fragment de séquence nucléotidique codant pour ladite protéine, une séquence anti-sens capable de s'hybrider spécifiquement avec une séquence nucléotidique codant pour ladite protéine, ou un anticorps dirigé contre ladite protéine, pour la fabrication d'un médicament destiné à traiter les maladies neurodégénératives et les néoplasmes.

18. Méthode de traitement des maladies neurodégénératives et des néoplasmes comprenant l'administration à un sujet nécessitant un tel traitement d'une quantité thérapeutiquement efficace d'une protéine purifiée de la famille ULIP, fragment polypeptidique ou dérivé biologiquement actif de celle-ci, une séquence ou fragment de séquence nucléotidique codant pour ladite protéine, une séquence anti-sens capable de s'hybrider spécifiquement avec une séquence nucléotidique codant pour ladite protéine, ou un anticorps dirigé contre ladite protéine, associée à un véhicule pharmaceutiquement acceptable.



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échelle de  
poids  
moléculaire +

66 kDa -

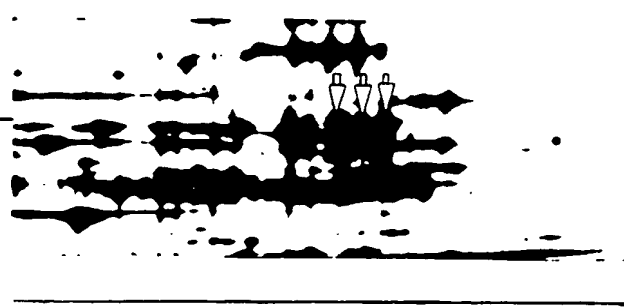


FIG. 1A

66 kDa -

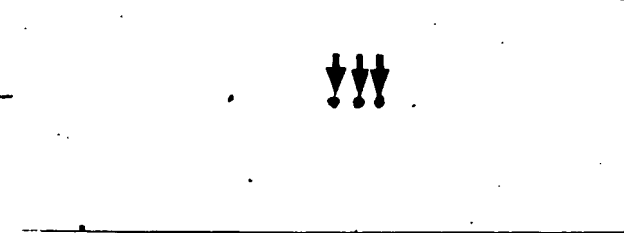
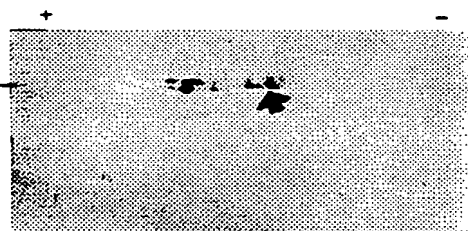


FIG. 1B

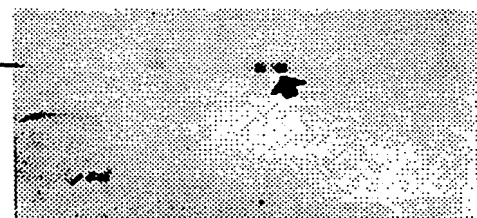
66 kDa -

échelle  
de poids  
moléculaire ↓



famille ULIP

66 kDa -



antigène CV<sub>2</sub> (POP 66/ULIP<sub>4</sub>)

FIG. 2A

FIG. 2B



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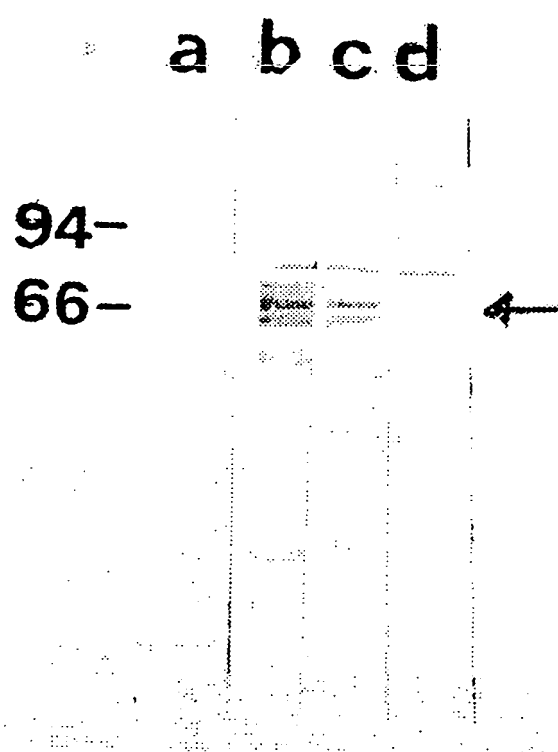


FIG. 3



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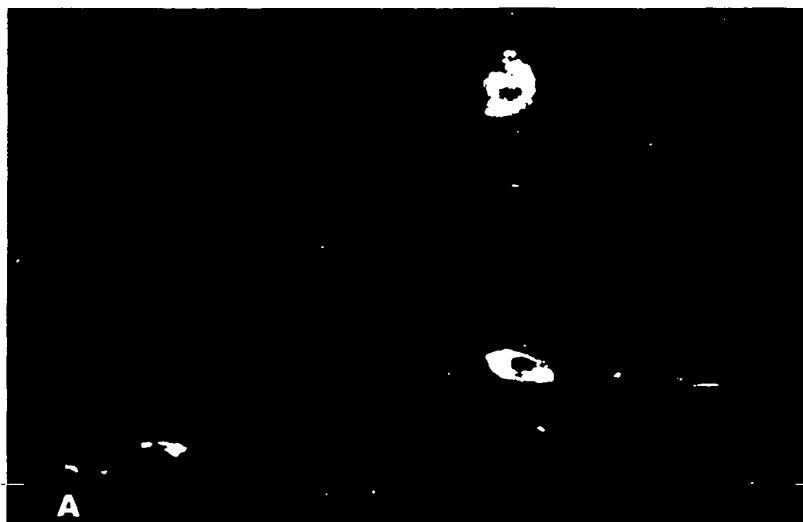


FIG. 4A



FIG. 4B



FIG. 4C





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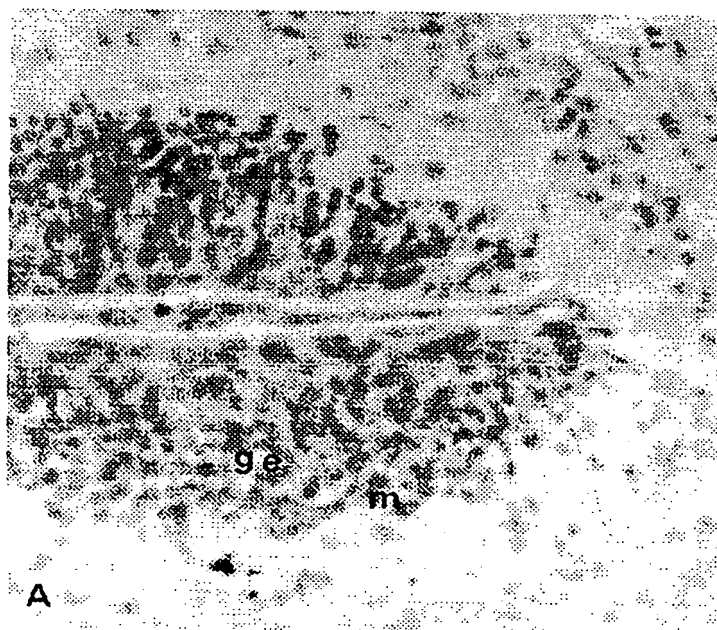


FIG. 5A

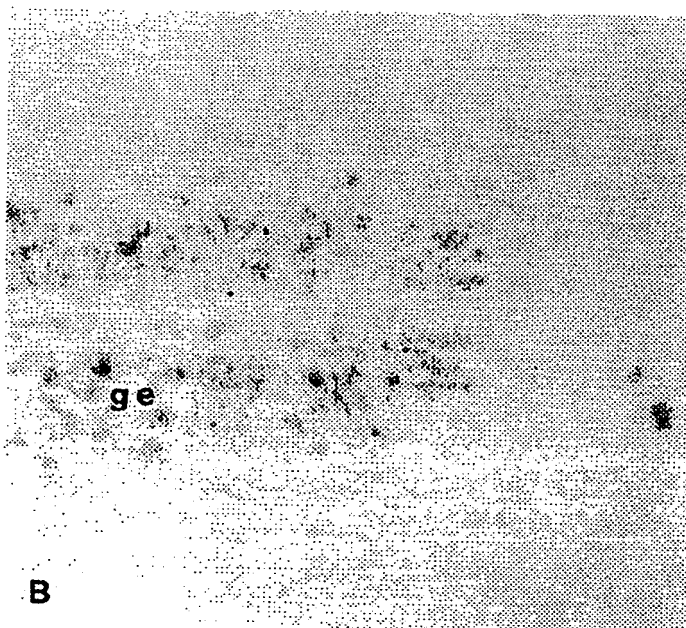


FIG. 5B



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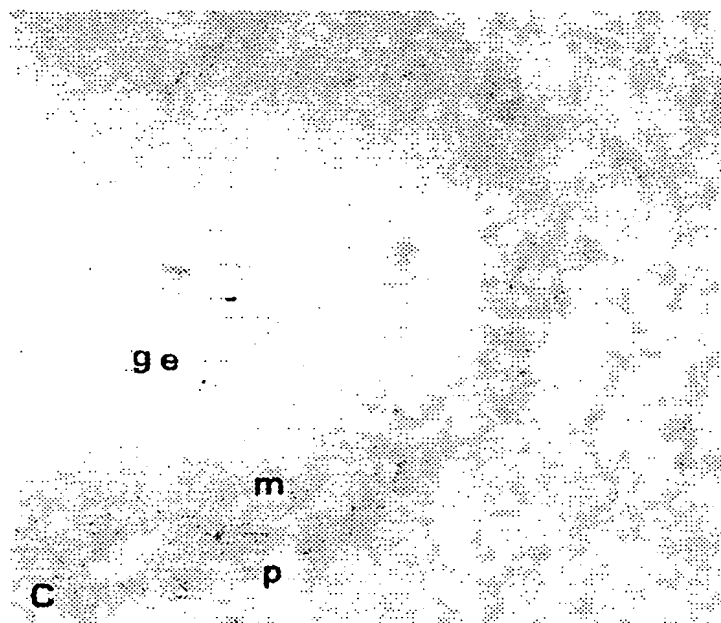


FIG. 5C

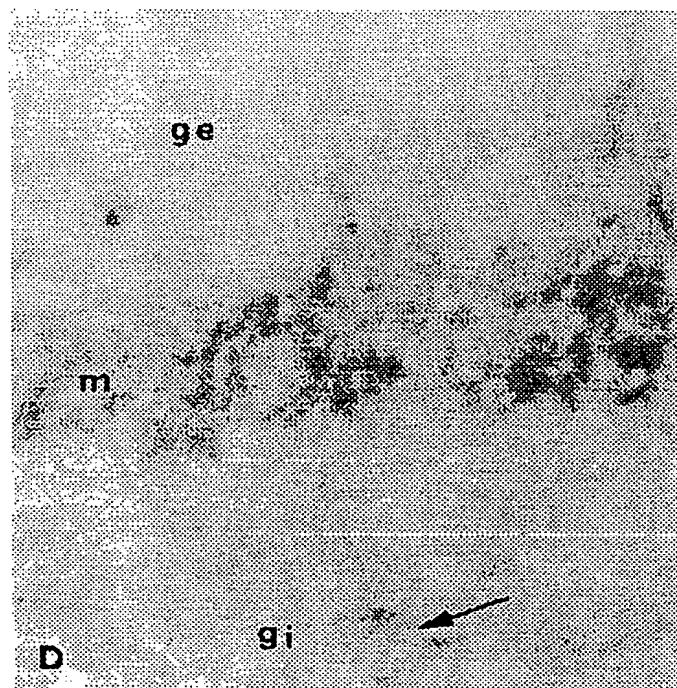


FIG. 5D



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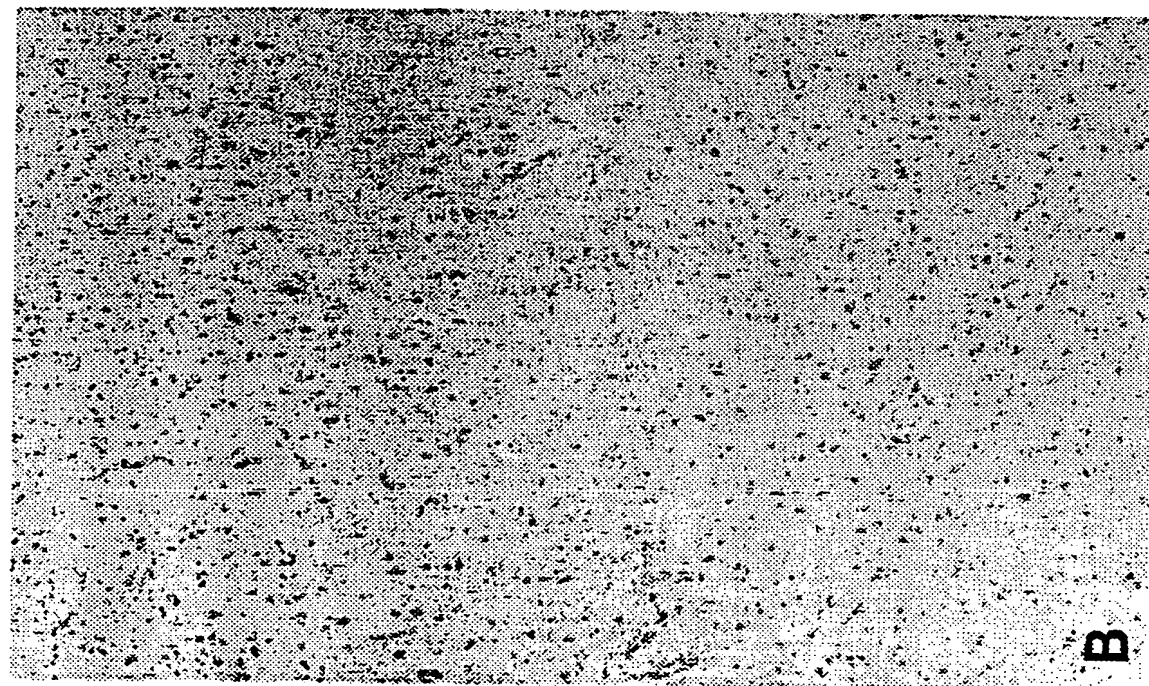


FIG. 6B

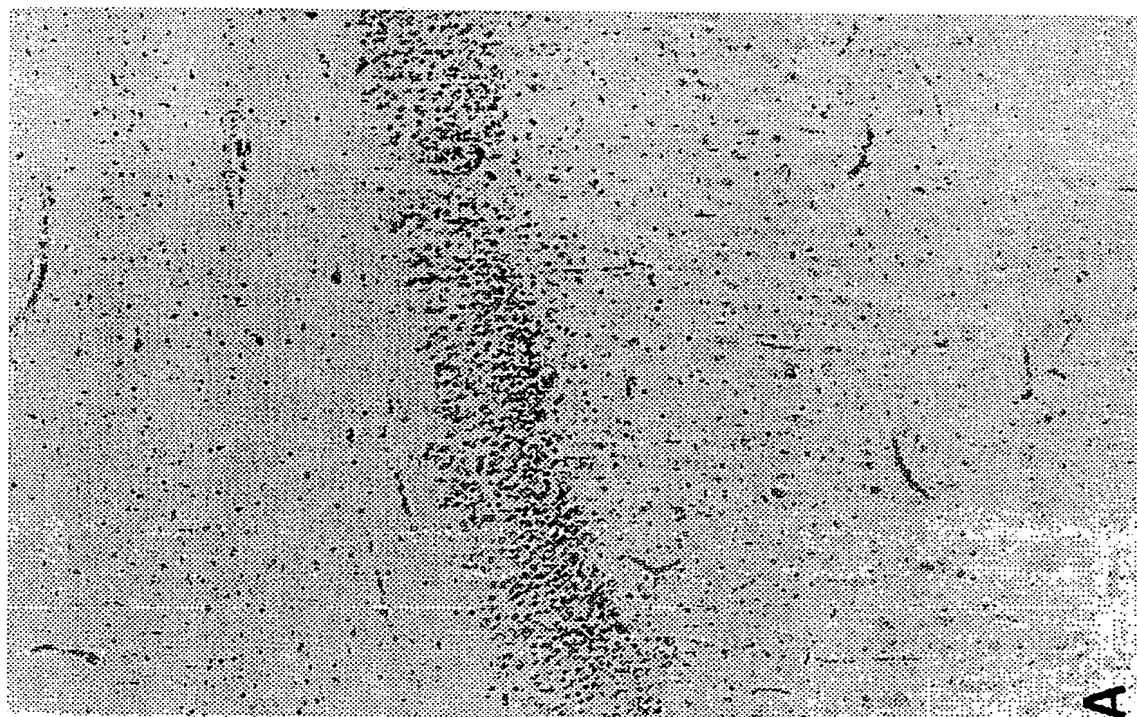


FIG. 6A



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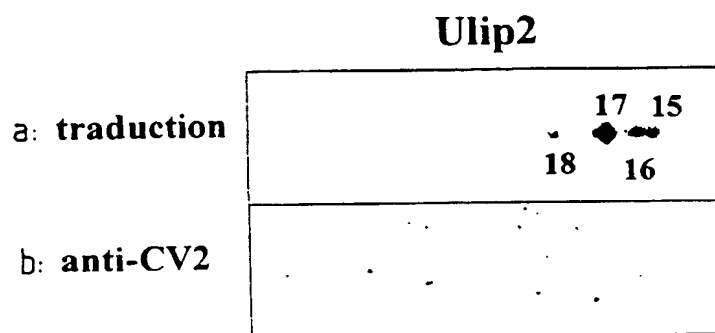


FIG. 7A

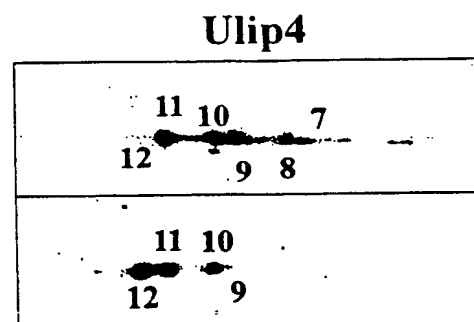


FIG. 7B

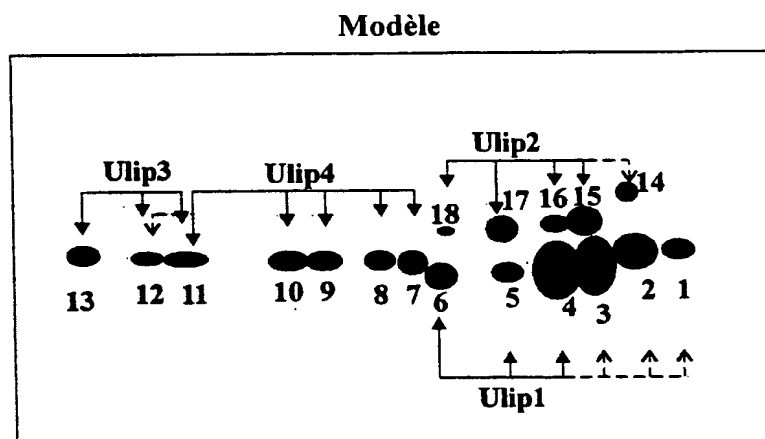
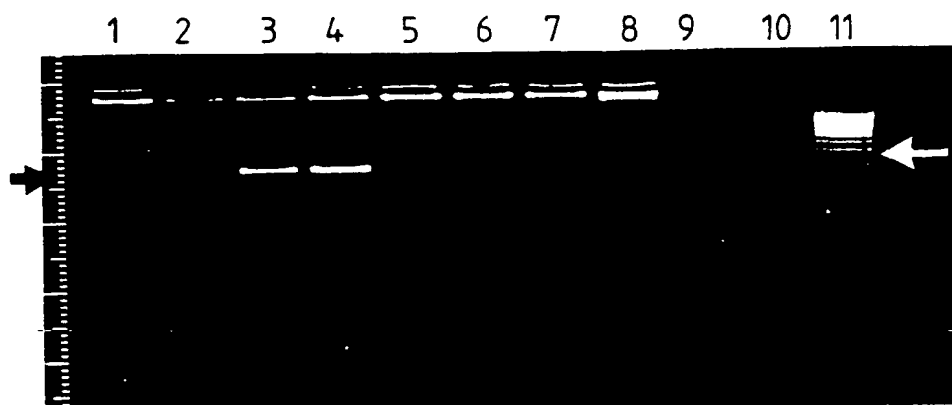
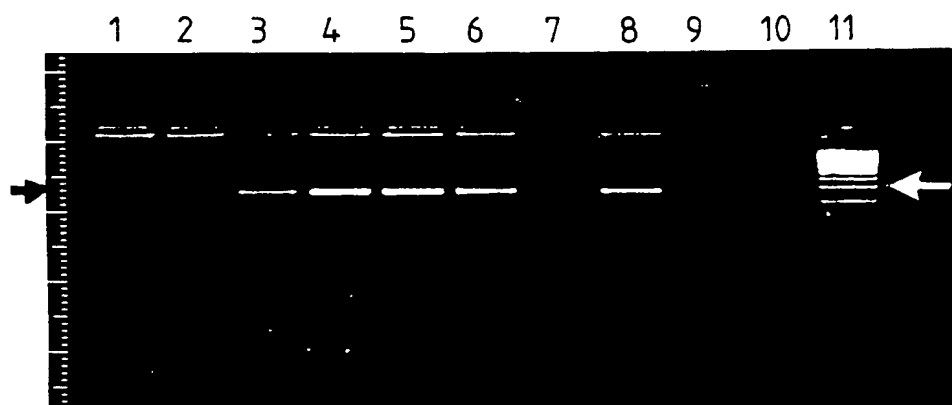


FIG. 7C





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FIG. 8AFIG. 8B



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**ulip2 souris**

cttcctccgccccccgagag	M	S	Y	Q	G	K	K	N	I	P	P	I	12
	ATG	TCT	TAT	CAG	GGG	AAG	AAA	AAT	ATT	CCA	CCC	ATC	58
T S D R L L I K G G K I V N D D Q S													30
ACG AGC GAT CGT CTT CTG ATC AAA GGT GGC AAG ATT GTG AAT GAT GAC CAG TCC													112
F Y A D I Y M E D G G L I K Q I G E N													48
TTC TAT GCA GAC ATA TAC ATG GAA GAT GGG TTG ATC AAG CAA ATA GGA GAA AAC													166
L I V P G G V K T I E A H S R M V I													66
CTG ATT GTA CCA GGA GGG GTG AAG ACC ATC GAA GCC CAC TCC AGA ATG GTG ATT													220
P G G I D V H T R F Q M P D Q G M T													84
CCC GGA GGA ATT GAC GTG CAT ACT CGC TTC CAG ATG CCT GAC CAG GGA ATG ACA													274
S A D D F F Q G T K A A L A G G T T													102
TCC GCT GAT GAC TTC CAG GGA ACC AAG GCG GCC CTG GCC GGG GGA ACC ACC													328
M I I D H V V P E P G T S L L A A F													120
ATG ATC ATT GAC CAT GTT GTT CCT GAG CCC GGG ACG AGC CTA TTG GCT GCC TTT													382
D Q W R E W A D S K S C C D Y S L H													138
GAT CAG TGG AGG GAG TGG GCT GAC AGC AAG TCC TGC TGT GAC TAT TCG CTG CAC													436
V D I T E W H K G I Q E E M E A L V													156
GTG GAC ATC ACT GAG TGG CAC AAG GGC ATC CAG GAG GAG ATG GAA GCT CTG GTG													490

**FIG.9**



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K D H G V N S F L V Y M A F K D R F	174
AAG GAC CAC GGG GTA AAC TCC TTC CTG TAC ATG GCT TTC AAA GAT CGA TTC	544
Q L T D S Q I Y E V L S V I R D I G	192
CAG CTG ACG GAT TCC CAG ATC TAT GAA GTG CTG AGC GTG ATC CGG GAT ATC GGT	598
A I A Q V H A E N G D I I A E A Q Q	210
GCC ATA GCT CAA GTC CAC GCA GAG AAT GGT GAC ATC ATT GCT GAG GCA CAG CAG	652
R I L D L G I T G P E G H V L S R P	228
AGG ATC CTG GAT CTG GGC ATC ACG GGC CCC GAG GGA CAC GTG TTG AGC CGG CCA	706
E E V E A E A V N R S I T I A N Q T	246
GAG GAG GTC GAG GCT GAA GCT GTG AAC CGG TCC ATC ACT ATT GCC AAC CAG ACC	760
N C P L Y V T K V M P K S A A E V I	264
AAC TGC CCT CTG TAT GTC ACC AAA GTG ATG CCC AAG AGT GCG GCT GAA GTC ATC	814
A Q A R K K G T V V Y G E P I T A S	282
GCT CAG GCA CGG AAG AAG GGA ACT GTG GTG TAT GGT GAG CCG ATC ACG GCC AGC	868
L G T D G S H Y W S K N W A K A A A	300
CTG GGG ACT GAT GGC TCT CAT TAC TGG AGC AAG AAC TGG GCC AAG GCT GCG GCC	922
F V T S P P L S P D P T T P D F L N	318
TTT GTC ACC TCC CCA CCC TTG AGC CCC GAC CCA ACC ACT CCA GAC TTT CTC AAC	976
S L L S C G D L Q V T G S A H C T F	336
TCG TTG CTG TCC TGT GGA GAC CTC CAA GTC ACT GGC AGT GCC CAC TGC ACC TTC	1030

FIG. 9 suite



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N T A Q K A V G K D N F T L I P E G 354  
 AAC ACT GCC CAG AAG GCT GTG GGG AAG GAC AAC TTC ACC TTG ATT CCC GAG GGC 1084  
  
 T N G T E E R M S V I W D K A V V T 372  
 ACC AAC GGC ACT GAG GAG CGG ATG TCT GTC ATT TGG GAT AAA GCT GTG GTC ACT 1138  
  
 G K M D E N Q F V A V T S T N A A K 390  
 GGG AAG ATG GAT GAG AAT CAG TTT GTG GCT GTG ACC AGC ACC AAC GCA GCC AAA 1192  
  
 V F N L Y P R K G R I S V G S D A D 408  
 GTC TTC AAC CTT TAC CCC CGG AAA GGT CGC ATC TCG GTG GGA TCT GAT GCT GAC 1246  
  
 L V I W D P D S V K T I S A K T H N 426  
 TTG GTC ATC TGG GAC CCT GAC AGT GTG AAG ACC ATC TCT GCC AAG ACA CAC AAC 1300  
  
 S A L E Y N I F E G M E C R G S P L 444  
 AGT GCT CTT GAG TAC AAC ATC TTT GAA GGC ATG GAG TGT CGC GGC TCC CCA CTG 1354  
  
 V V I S Q G K I V L E D G T L H V T 462  
 GTG GTC ATC AGC CAG GGC AAG ATT GTC CTG GAG GAC GGC ACA CTT CAT GTC ACT 1408  
  
 E G S G R Y I P R K P F P D F V Y K 480  
 GAA GGC TCA GGA CGC TAC ATT CCC CGG AAG CCC TTC CCT GAC TTT GTG TAC AAA 1462  
  
 R I K A R S R L A E L R G V P R G L 498  
 CGC ATC AAA GCA AGG AGC AGG CTG GCT GAG CTG AGA GGG GTC CCT CGT GGC CTG 1516  
  
 Y D G G P V C E V S V T P K T V T P A 516  
 TAT GAC GGA CCG GTA TGC GAG GTG TCT GTG ACG CCC AAG ACG GTG ACT CCA GCC 1570

FIG. 9 suite





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S S A K T S P A K Q Q CAG CAG GCA CCA CCT GTT CGG AAC CTG 534  
TCA TCA GCT AAG ACA TCC CCT GCC AAG CAG CAG GCA CCA CCT GTT CGG AAC CTG 1624

H Q S G F S L S G A Q I D D N I P R 552  
CAC CAG TCT GGA TTC AGC TTG TCT TCT GGT GGT GCT CAG ATT GAC GAC AAC ATT CCC CGC 1678

R T T Q R I V A P P G G GGT GGT GGC CGT GCC AAC ATC ACC AGC 570  
CGC ACC ACC CAG CGC ATC GTG GCA CCC CCT GGT GGT GGC CGT GCC AAC ATC ACC AGC 1732

L G \* 573  
CTG GGC TAA agccccctagccctgcaggccacttggggatggggatgggacacctgaggacattctga 1800

gacttcctttcttccat

## FIG. 9 suite



# Ulip3 souris

gctgtctgtcttcagcgccctctctgcctgcctctctccctctctccctctcttgcacggcggc 72

gggtgcaggcagccgggagcagcgggcgggcgaggcagcggtgggagccgagcttctg 144

tctttcttcatccctccctggccttgtcgcgctctcacgagtagcgccgggagagaccgggtag 216

	M	S	H	Q
agcgccaggcagacgttagttccagcgccggcgagggtccagaggggcc	ATG	TCT	CAT	CAG
				281

G	K	K	S	I	P	H	I	T	S	D	R	L	L	I	R	G	G	22
GGG	AAG	AAG	AGC	ATC	CCG	CAC	ATC	ACC	AGT	GAC	CGG	CTC	CTC	ATC	AGA	GGT	GGA	335

R	I	I	N	D	D	Q	S	F	Y	A	D	V	Y	L	E	D	G	40
CGC	ATC	ATC	AAT	GAT	GAC	CAG	TCC	TTC	TAC	GCC	GAT	GTC	TAC	CTA	GAA	GAT	GGA	389

L	I	K	Q	I	G	E	N	L	I	V	P	G	G	V	K	T	I	58
CTC	ATA	AAA	CAA	ATA	GGA	GAG	AAC	CTG	ATT	GTT	CCT	GGT	GGA	GTG	AAG	ACC	ATC	443

E	A	N	G	R	M	V	I	P	G	G	I	D	V	N	T	Y	L	76
GGAG	GCG	AAT	GGC	CGA	ATG	GTC	ATT	CCC	GGT	GGC	ATT	GAT	GTC	AAC	ACT	TAC	CTG	497

Q K P S Q G M T S A D D F Q G T K 94  
CAG AAG CCC TCC CAG GGC ATG ACC TCG GCT GAT GAC TTC TTC CAG GGC ACT AAA 551

[illegible][illegible]

FIG. 10



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S C C D Y S L H V D I T S W Y D G V 148  
 TCC TGC TGT GAC TAT TCC CTC CAC GTG GAC ATC ACA AGC TGG TAT GAT GGT GTT 713  
  
 R E E L E V L V Q D K G V N S F Q V 166  
 CGG GAA GAG CTG GAG GTG CTG GTG CAG GAC AAA GGT GTC AAC TCC TTC CAA GTC 767  
  
 Y M A Y K D L Y Q M S D S Q L Y E A 184  
 TAC ATG GCG TAT AAG GAC CTG TAC TAC CAG ATG TCT GAC AGC CAG CTG TAT GAA GCC 821  
  
 F T F L K G L G A V I L V H A E N G 202  
 TTC ACC TTC CTT AAG GGT TTG GGA GCT GTG ATC TTA GTC CAT GCA GAA AAT GGA 875  
  
 D L I A Q E Q K R I L E M G I T G P 220  
 GAT TTG ATA GCT CAG GAA CAA AAA CCG ATC CTG GAG ATG GGC ATC ACG GGT CCC 929  
  
 E G H A L S R P E E L E A E A V F R 238  
 GAG GGT CAT GCT CTG AGC AGA CCC GAG GAG CTG GAG GCC GAG GCT GTG TTC CGG 983  
  
 A I A I A G R I I N C P V Y I T K V M 256  
 GCT ATT GCC ATT GCA GGC CGG ATC AAT TGC CCT GTG TAC ATC ACC AAG GTC ATG 1037  
  
 S K S A A D I I A L A R K K G P L V 274  
 AGC AAG AGT GCA GCG GAC ATC ATC GCA CTG GCC AGG AAG AAA GGC CCT CTT GTC 1091  
  
 F G E P I A A S L G T D G T H Y W S 292  
 TTC GGT GAG CCC ATA GCC GCC AGC CTG GGA ACC GAT GGC ACC CAC TAC TGG AGC 1145  
  
 K N W A K A A A F V T S P P L S P D 310  
 AAG AAC TGG GCC AAG GCA GCT GCA TTT GTG ACT TCC CCT CCC CTG AGC CCA GAC 1199

FIG.10suite



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P T T P D Y L T S L L A C G D L Q V 328  
 CCC ACC ACT CCT GAC TAC TTG ACC TCC TTG CTG GCC TGT GGA GAC TTG CAG GTC 1253  
  
 T G S G H C P Y S I A Q K A V G K D 346  
 ACA GGT AGT GGC CAC TGT CCC TAC AGT ATT GCT CAG AAG GCT GTG GGC AAG GAC 1307  
  
 N F T L I P E G V N G I E E R M T V 364  
 AAC TTC ACT CTG ATC CCT GAG GGT GTC AAT GGT ATA GAA GAG CGG ATG ACC GTT 1361  
  
 V W D K A V A T G K M D E N Q F V A 382  
 GTC TGG GAC AAG GCA GTG GCT ACT GGC AAG ATG GAT GAG AAC CAG TTT GTA GCC 1415  
  
 V T S T N A A K I F N L Y P R K G R 400  
 GTC ACC AGC ACC AAC GCA GCC AAG ATC TTC AAC CTG TAC CCG AGG AAA GGT CGG 1469  
  
 I A V G S D A D V V I W D P D K M K 418  
 ATC GCT GTG GGC TCC GAT GCT GAT GAC GTA GTC ATC TGG GAC CCA GAT AAG ATG AAG 1523  
  
 T I T A K S H K S T V E Y N I F E G 436  
 ACC ATA ACA GCC AAA AGC CAT AAA TCA ACT GTG GAG TAC AAC ATC TTT GAG GGC 1577  
  
 M E C H G S P L V V I S Q G K I V F 454  
 ATG GAG TGC CAC GGC TCC CCC CTG GTG GTC ATC AGT CAG GGC AAG ATT GTC TTT 1631  
  
 E D G N I S V S K G M G R F I P R K 472  
 GAG GAT GGA AAC ATC AGT GTC AGC AAG GGC ATG GGC CGC TTC ATC CCT CGG AAG 1685  
  
 P F P E H L Y Q R V R I R S K V F G 490  
 CCA TTC CCA GAG CAT CTC TAC CAG CGT GTC AGG ATC AGA AGC AAG GTT TTC GGG 1739

FIG.10 suite





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L H S V S R G G M Y D G P V Y E V P A 508  
 TTG CAT AGT GTT TCC AGG GGC ATG TAC GAT GAT GGG CCT GTG TAC GAG GTG CCA GCT 1793  
  
 T P K H A A P A P S A E S S P S K H 526  
 ACA CCC AAA CAT GCT GCT CCT GCT CCT TCT TCT GCC GAA TCC TCG CCT TCT AAA CAC 1847  
  
 Q P P CCA CCC ATC CGG AAC CTC CAC CAG TCC AAC TTC AGC TTA TCA GGT GCC 1901  
 CAA CCC CCA CCC ATC CGG AAC CTC CAC CAG TCC AAC TTC AGC TTA TCA GGT GCC 1901  
  
 Q I D D N N P R R T G H R I V A P P 562  
 CAG ATA GAT GAC AAC AAT CCA AGG CGT ACA GGC CAC CGC ATT GTG GCG CCC CCT 1955  
  
 G G R S N I T S L G \* 573  
 GGT GGC CGC TCC AAC ATC ACC AGC CTC GGT TGA cctcagatgagccagatatgcaagagt 2015  
  
 gaaggattatgggaaaacgtccattccttttccgtgttttgaagccacagtttagttggtactgacgga 2087  
  
 ggggagattgagcgatgctcttccctctctgttaggaagaagtgttagtggtgttgcctgga 2159  
  
 agtccctcgcccacagtgtgtgttcacaccgactccacctcagagcatggtgccgtccgttttcccttccta 2231  
  
 gtgaccccggttagcatcgtcctatactgttccctccactcctccatgacctctgagtatgg 2297

FIG.10 suite



## Ulip4 souris

gctgactaatatgcttaaatcagcgggtgccacgtctggtcggtacgtccacgcccgcagccccctacc 72  
 gagacactcagcccgccgtgtatcagg ATG TCC TTC CAA GGC AAG AAG AGC ATT CCC 10  
 131  
 R I T S D R L L I K G G K I V N D D 28  
 CGG ATA ACG AGC GAC CGC CTT CTC ATC AAA GGT GGG AAG ATT GTG AAC GAT GAC 185  
 Q S F H A D L Y V E D G L I K Q I G 46  
 CAG TCC TTT CAT GCT GAT CTG TAT GTG GAA GAC GGT CTG ATT AAA CAA ATT GGA 239  
 E N L I V P G G I K T I D A H G L M 64  
 GAA AAT CTC ATC GTC CCT GGG GGC ATC AAA ACC ATC GAT GCT CAT GGC CTG ATG 293  
 V L P G G V D V H T R L Q M P V M G 82  
 GTG CTG CCT GGG GGA GTT GAC GTT CAC ACC CGG CTG CAG ATG CCT GTG ATG GGC 347  
 M T P A D D F C Q G T K A A L A G G 100  
 ATG ACC CCA GCT GAT GAT TTC TGT CAG GGC ACC AAG GCG GCT CTA GCA GGC GGG 401  
 T T M I L D H V F P D A G V S L L A 118  
 ACC ACC ATG ATA TTG GAC CAT GTG TTT CCT GAC GCT GGT GTG AGC CTG CTG GCA 455  
 A Y E Q W R D G A D S A A C C D Y S 136  
 GCC TAT GAG CAG TGG CGG GAC GGA GCA GAC AGC GCG GCC TGC TGT GAC TAC TCC 509  
 L H V D I P R W H E S T K E E L E A 154  
 TTA CAT GTG GAC ATT CCT CGC TGG CAC GAG AGC ACC AAA GAA GAG CTG GAG GCC 563

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FIG.11



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L V R D K G V N S F L V F M A Y K D 172  
 CTA GTC AGG GAC AAA GGT GTG AAC TCC TTC CTG GTC TTC ATG GCA TAC AAG GAC 617  
  
 R C Q C T D G G Q I Y E I F S L I R D 190  
 AGG TGC CAG TGT ACT GAC GGT CAG ATA TAT GAA ATC TTC AGC CTC ATC CGG GAC 671  
  
 L G A V A Q V H A E N G D I V E E 208  
 CTG GGA GCT GTG GCC CAG GTG CAC GCA GAA AAT GGG GAC ATC GTG GAG GAG GAA 725  
  
 Q K R L L E Q G I T G P E G H V L S 226  
 CAG AAG CGC CTG CTG GAG CAA GGC ATC ACT GGT CCT GAG GGC CAT GTG CTC AGC 779  
  
 H P E E V A A E A V Y R A V T I A K 244  
 CAC CCA GAA GAG GTA GAG GCC GAG GCT GTG TAC AGA GCA GTC ACC ATT GCC AAG 833  
  
 Q A N C P L Y V T K V M S K G A A D 262  
 CAG GCC AAC TGC CCA CTA TAC GTC ACC AAG GTG ATG AGC AAG GGT GCA GCT GAC 887  
  
 M V A Q A K R R G V V V F G E P I T 280  
 ATG GTT GCC CAA GCC AAG CGC AGG GGG GTG GTC GTC TTT GGG GAA CCT ATC ACT 941  
  
 A S L G T D G S H Y W S K N W A K A 298  
 GCC AGC CTG GGC ACT GAT GGC TCA CAC TAC TGG AGC AAG AAC TGG GCC AAG GCT 995  
  
 A A F V T S P P I N P D P T T A D H 316  
 GCA GCC TTT GTC ACT TCA CCC CCT ATC AAC CCG GAC CCT ACT ACT GCA GAC CAC 1049  
  
 L T S L L S S G D L Q V T G S A H C 334  
 CTC ACC TCT CTG CTG TCC AGT GGG GAC CTC CAG GTG ACA GGC AGT GCC CAC TGC 1103

FIG.11 suite



2

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T	F	T	T	A	Q	K	A	V	G	K	D	N	F	T	L	I	P	352
ACC	TTC	ACT	ACT	GCC	CAG	AAG	GCT	GTT	GGC	AAA	GAC	AAC	TTC	ACA	CTG	ATC	CCC	1157
E	V	V	N	G	I	E	E	R	M	S	V	V	W	E	K	C	V	370
GAG	GTA	GTC	AAC	GGT	ATA	GAA	GAG	CGC	ATG	TCT	GTG	GTC	TGG	GAG	AAA	TGT	GTG	1211
A	S	G	K	M	D	E	N	E	F	V	A	V	T	S	T	N	A	388
GCT	TCA	GGG	AAA	ATG	GAC	GAG	AAT	GAG	TTC	GTT	GCC	GTG	ACC	AGC	ACA	AAT	GCT	1265
A	K	I	F	N	F	Y	P	R	K	G	R	V	A	V	G	S	D	406
GCC	AAA	ATC	TTC	AAT	TTT	TAC	CCC	AGG	AAG	GGG	CGT	GTG	GCC	GTG	GGC	TCT	GAT	1319
A	D	L	V	I	W	N	P	R	A	T	K	V	I	S	A	K	S	424
GCT	GAC	CTG	GTC	ATC	TGG	AAC	CCC	AGG	GCC	ACG	AAA	GTC	ATC	TCT	GCC	AAG	AGC	1373
H	N	L	N	V	E	Y	N	I	F	E	G	V	E	C	R	G	V	442
CAT	AAC	CTG	AAT	GTA	GAG	TAC	AAC	ATC	TTT	GAA	GGA	GTG	GAG	TGC	CGA	GGA	GTG	1427
P	T	V	V	I	S	Q	G	R	V	V	L	E	D	G	N	L	L	460
CCC	ACG	GTG	GTC	ATA	AGT	CAG	GGC	AGA	GTG	GTG	CTG	GAG	GAC	GGA	AAC	CTG	CTT	1481
V	T	P	G	A	G	R	F	I	P	R	K	T	F	P	D	F	V	478
GTC	ACT	CCA	GGG	GCT	GGC	CGC	TTC	ATT	CCC	CGG	AAG	ACG	TTC	CCG	GAC	TTT	GTC	1535
Y	K	R	I	K	A	R	N	R	L	A	E	I	H	G	V	P	R	496
TAT	AAG	AGG	ATA	AAG	GCT	CGC	AAC	AGG	CTA	GCA	GAG	ATC	CAC	GGT	GTG	CCT	CGA	1589

FIG.11 suite





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G L Y D G G G C C T G T G C A T G A A G T G A T G T T A C C T G C C A A G C C A G G A A G T S 514  
GGC CTG TAC GAC GAC GGC CCT GGT GTG ATG TTA CCT GGC AAG CCA GGA AGT 1643

G T Q A R A S C S G K I S V P P V R 532  
GGC ACA CAG GCC CGT GCA TCC TGT TCA GGC AAG ATC TCA GTG CCA CCC GTG CGC 1697

N L H Q S G F S L S G S Q A D D H I 550  
AAC CTG CAC CAG TCG GGG TTC AGC CTA TCT GGC TCT CAG GCT GAC GAT CAC ATT 1751

A R R T A Q K I M A P P G G R S N I 568  
GCC AGA CGT ACG GCT CAG AAG ATC ATG GCA CCC GGA GGA CGC TCC AAC ATC 1805

T S L S \* 573  
ACG TCT CTT TCC TAG acttgggggtcttggcaagctggtgctgtctccactggcagggtgtgtgggac 1871

gactcacgtcagttagctccttcctttgttagattgttattgtgaaaggg 1920

## FIG.11 suite



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[illegible]

FIG. 12



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L	V	K	E	K	G	V	N	S	F	L	V	F	M	A	Y	K	D	172
CTG	GTC	AAG	GAG	AAG	GGT	GTG	AAC	TCC	TTC	CTG	GTC	TTC	ATG	GCA	TAC	AAG	GAC	547
R	C	Q	C	S	D	S	Q	M	Y	E	I	F	S	I	I	R	D	190
CGG	TGC	CAG	TGC	AGC	GAC	AGC	CAG	ATG	TAC	GAG	ATC	TTC	AGC	ATC	ATC	CGG	GAC	601
L	G	A	L	A	Q	V	H	A	E	N	G	D	I	V	E	E	E	208
CTG	GGG	GCC	TTG	GCC	CAG	GTG	CAC	GCT	GAG	AAC	GGG	GAC	ATC	GTG	GAG	GAG	GAG	655
Q	K	R	L	L	E	L	G	I	T	G	P	E	G	H	V	L	S	226
CAG	AAG	CGG	TTG	CTG	GAG	CTC	GGC	ATC	ACT	GGC	CCC	GAG	GGC	CAC	GTG	CTC	AGC	709
H	P	E	E	V	E	A	E	A	V	Y	R	A	V	T	I	A	K	244
CAC	CCC	GAG	GAG	GTG	GAG	GCT	GAG	GCG	GTG	TAC	CGA	GCT	GTC	ACC	ATC	GCC	AAG	763
Q	A	N	C	P	L	Y	V	T	K	V	M	S	K	G	A	A	D	262
CAG	GCA	AAC	TGC	CCG	CTG	TAC	TAC	ACC	AAG	GTG	ATG	AGC	AAG	GGG	GCG	GCC	GAC	817
A	I	A	Q	A	K	R	R	G	V	V	V	F	G	E	P	I	T	280
GCC	ATC	GCT	CAG	GCC	AAG	CGC	AGA	GGG	GTG	GTC	GTG	TTT	GGG	GAG	CCC	ATC	ACC	871
A	S	L	G	T	D	G	S	H	Y	W	S	K	N	W	A	K	A	298
GCC	AGC	CTG	GGC	ACC	GAC	GGT	TCA	CAC	TAC	TGG	AGC	AAG	AAC	TGG	GCC	AAG	GCT	925
A	A	F	V	T	S	P	P	V	N	P	D	P	T	T	A	D	H	316
GCA	GCC	TTC	GTC	ACA	TCA	CCC	CCT	GTC	AAC	CCA	GAC	CCC	ACC	ACG	GCA	GAC	CAC	979
L	T	C	L	L	S	S	G	D	L	Q	V	T	G	S	A	H	C	334
CTC	ACC	TGC	TTG	CTG	TCC	AGC	GGG	GAC	CTC	CAG	GTG	ACA	GGC	AGC	GCC	CAC	TGC	1033

FIG.12 suite



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T F T T A Q K A V G K D N F A L I P 352  
 ACC TTC ACC ACT GCC CAG AAG GCT GTG GGC AAG GAC AAC TTC GCG CTG ATC CCC 1087  
  
 E G T N G I E E R M S M V W E K C V 370  
 GAG GGC ACC AAC GGC ATT GAG GAG CGC ATG TCG ATG GTC TGG GAG AAA TGT GTG 1141  
  
 A S G K M D E N E F V A V T S T N A 388  
 GCC TCT GGG AAG ATG GAC GAG AAT GAG TTC GTC GCG GTG ACC AGT ACA AAT GCT 1195  
  
 A K I F N F Y P R K G R V A V G S D 406  
 GCC AAA ATC TTC AAT TTT TAC CCA AGG AAG GGG CGA GTG GCT GTG GGC TCT GAC 1249  
  
 A D L V I W N P K A T K I I S A K T 424  
 GCT GAC CTG GTC ATA TGG AAC CCC AAG GCC ACC AAG ATC ATC TCT GCC AAG ACC 1303  
  
 H N L N V E Y N I F E G V E C R G A 442  
 CAC AAT CTG AAC GTG GAG TAC AAC ATC TTC GAG GGA GTG GAG TGC CGG GGA GCG 1357  
  
 P A V V I S Q G R V A L E D G K M F 460  
 CCT GCC GTG GTC ATA AGT CAG GGC CGA GTG GCG CTG GAG GAC GCG AAG ATG TTT 1411  
  
 V T P G A G R F V P R K T F P D F V 478  
 GTC ACC CCG GGG GCG CGC TTC GTC CCT CCG AAA ACA TTC CCG GAC TTT GTC 1465  
  
 Y K R I K A R N R L A E I H G V P R 496  
 TAC AAG AGG ATC AAA GCT CGC AAC AGG CTG GCG GAG ATC CAC GGT GTG CCC CGT 1519

FIG.12suite





[illegible]

## FIG.12 suite



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## INTERNATIONAL SEARCH REPORT

International Application No

PCT/FR 98/00328

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/12 C07K14/47 C07K16/18 G01N33/574 C12Q1/68  
A61K38/17 A61K48/00 A61K39/395

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K G01N C12Q A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JÉRÔME HONNORAT ET AL.: "Antibodies to a subpopulation of glial cells and a 66kDa developmental protein in patients with paraneoplastic neurological syndromes" JOURNAL OF NEUROLOGY, NEUROSURGERY AND PSYCHIATRY, vol. 61, 1996, pages 270-278, XP002045119 cited in the application see page 270, left-hand column, paragraph 2 see page 272, right-hand column, paragraph 2 see page 276, right-hand column, paragraph 2 see page 277, right-hand column, paragraph 1 --- -/--	1,2,7-18



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

## \* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance  
"E" earlier document but published on or after the international filing date  
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)  
"O" document referring to an oral disclosure, use, exhibition or other means  
"P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention  
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone  
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.  
"&" document member of the same patent family

Date of the actual completion of the international search

30 June 1998

Date of mailing of the international search report

23.07.98

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Authorized officer

Montero Lopez, B

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/FR 98/00328

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>TAMARA BYK ET AL.: "Identification and molecular characterization of Unc-33-like phosphoprotein (Ulip), a putative mammalian homolog of the axonal guidance-associated unc-33 gene product" JOURNAL OF NEUROSCIENCE, vol. 16, no. 2, 15 January 1996, pages 688-701, XP002045120 cited in the application see abstract see page 690, left-hand column, paragraph 8 - page 693, left-hand column, paragraph 2 see page 693, right-hand column, paragraph 3 - page 694, left-hand column, paragraph 1 see page 697, right-hand column, paragraph 2 - page 701, right-hand column, last paragraph</p>	1,3,5-7, 10,11, 15,17,18
X	<p>YOSHIO GOSHIMA ET AL.: "Collapsin-induced growth cone collapse mediated by an intracellular protein related to UNC-33" NATURE, vol. 376, no. 6540, 10 August 1995, LONDON GB, pages 509-514, XP002069770 cited in the application see page 509, right-hand column, paragraph 1 - page 514, left-hand column, last paragraph; figure 1C</p>	1,3-7
X	<p>Base de données EMBL Numéro d'accès Q62950; 1 November 1996 WANG, L. ET AL. XP002045123 see the whole document</p>	1
A	<p>NAOKI HAMAJIMA ET AL.: "A novel gene family defined by human dihydropyrimidinase and three related proteins with differential tissue distribution" GENE, vol. 180, no. 1/2, 1996, AMSTERDAM NL, pages 157-163, XP002045121 see the whole document</p>	1-7
A	<p>Base de données EMBL entrée HS901245 Numéro d'accès H85901; 22 November 1995 HILLIER L. ET AL.: "The WashU-Merck EST Project." XP002045124 see the whole document</p>	3,4
	-/--	

## INTERNATIONAL SEARCH REPORT

Intern. Application No

PCT/FR 98/00328

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>BYK T ET AL: "The Ulips: A growing protein family related to the axonal guidance associated UNC-33 protein." 26TH ANNUAL MEETING OF THE SOCIETY FOR NEUROSCIENCE, WASHINGTON, D.C., USA, NOVEMBER 16-21, 1996. SOCIETY FOR NEUROSCIENCE ABSTRACTS 22 (1-3). 1996. 1713. ISSN: 0190-5295, XP002045122 * abrégé 676.9 *</p> <p>---</p>	1-18
A	<p>JANE E. MINTURN ET AL.: "TOAD-64, a gene expressed early in neuronal differentiation in the rat, is related to unc-33, a C. elegans gene involved in axon outgrowth" THE JOURNAL OF NEUROSCIENCE, vol. 15, no. 10, October 1995, pages 6757-6766, XP002069771 see the whole document</p> <p>---</p>	1-18
P,X	<p>Base de données Trrod Numéro d'accès 008553; 1 July 1997 BYK T. ET AL.: "ULIP2 Protein" XP002069773 see the whole document</p> <p>---</p>	1,7,10, 15
P,X	<p>Base de données Trrod Numéro d'accès 008554; 1 July 1997 BYK T. ET AL.: "ULIP3 Protein" XP002069774 see the whole document</p> <p>---</p>	1,7,10, 15
P,X	<p>Base de données Trrod Numéro d'accès 008886; 1 July 1997 BYK T. ET AL.: "ULIP4 Protein" XP002069775 see the whole document</p> <p>---</p>	1,7,10, 15
P,X	<p>GAETANO, CARLO ET AL: "Identification and characterization of a retinoic acid-regulated human homolog of the unc - 33 - like phosphoprotein gene (hUlip) from neuroblastoma cells" J. BIOL. CHEM. (1997), 272(18), 12195-12201 CODEN: JBCHA3; ISSN: 0021-9258, 1997, XP002069772 see page 12195, right-hand column, paragraph 2 see page 12196, right-hand column, paragraph 2 - page 12201, left-hand column, paragraph 2</p> <p>-----</p>	1-18

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/FR 98/00328

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
  
Although claim 18 concerns a method for the treatment of the human/animal body, the search was carried out on the basis of the effects attributed to the products.
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6 4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

## RAPPORT DE RECHERCHE INTERNATIONALE

(article 18 et règles 43 et 44 du PCT)

Référence du dossier du déposant ou du mandataire <b>BET 98/0077</b>	<b>POUR SUITE A DONNER</b> voir la notification de transmission du rapport de recherche internationale (formulaire PCT/ISA/220) et, le cas échéant, le point 5 ci-après	
Demande internationale n° <b>PCT/ FR 98/ 00328</b>	Date du dépôt international (jour/mois/année) <b>19/02/1998</b>	(Date de priorité (la plus ancienne) (jour/mois/année) <b>19/02/1997</b>
Déposant <b>INSTITUT NATIONAL DE LA SANTE ET DE ... et al.</b>		

Le présent rapport de recherche internationale, établi par l'administration chargée de la recherche internationale, est transmis au déposant conformément à l'article 18. Une copie en est transmise au Bureau international.

Ce rapport de recherche internationale comprend 5 feuilles.

☒ Il est aussi accompagné d'une copie de chaque document relatif à l'état de la technique qui y est cité.

1. ☒ Il a été estimé que certaines revendications ne pouvaient pas faire l'objet d'une recherche (voir le cadre I).

2. ☐ Il y a absence d'unité de l'invention (voir le cadre II).

3. ☒ La demande internationale contient la divulgation d'un listage de séquence de nucléotides ou d'acides aminés et la recherche internationale a été effectuée sur la base du listage de séquence

☒ déposé avec la demande internationale

☐ fourni par le déposant séparément de la demande internationale

☐ sans être accompagnée d'une déclaration selon laquelle il n'inclut pas d'éléments allant au-delà de la divulgation faite dans la demande internationale telle qu'elle a été déposée.

☐ transcrit par l'administration

4. En ce qui concerne le titre, ☒ le texte est approuvé tel qu'il a été remis par le déposant.

☐ Le texte a été établi par l'administration et a la teneur suivante:

5. En ce qui concerne l'abrégé,

☒ le texte est approuvé tel qu'il a été remis par le déposant

☐ le texte (reproduit dans le cadre III) a été établi par l'administration conformément à la règle 38.2b). Le déposant peut présenter des observations à l'administration dans un délai d'un mois à compter de la date d'expédition du présent rapport de recherche internationale.

6. La figure des dessins à publier avec l'abrégé est la suivante:

Figure n°        ☐ suggérée par le déposant.

☐ parce que le déposant n'a pas suggéré de figure.

☐ parce que cette figure caractérise mieux l'invention.

☒ Aucune des figures n'est à publier.





**Cadre I Observations - lorsqu'il a été estimé que certaines revendications ne pouvaient pas faire l'objet d'une recherche (suite du point 1 de la première feuille)**

Conformément à l'article 17.2)a), certaines revendications n'ont pas fait l'objet d'une recherche pour les motifs suivants:

1. ☒ Les revendications n<sup>os</sup> se rapportent à un objet à l'égard duquel l'administration n'est pas tenue de procéder à la recherche, à savoir:  
  
Bien que la revendication 18 concerne une méthode de traitement du corps humain/animal, la recherche a été effectuée et basée sur les effets imputés aux produits.
2. ☐ Les revendications n<sup>os</sup> se rapportent à des parties de la demande internationale qui ne remplissent pas suffisamment les conditions prescrites pour qu'une recherche significative puisse être effectuée, en particulier:
3. ☐ Les revendications n<sup>os</sup> sont des revendications dépendantes et ne sont pas rédigées conformément aux dispositions de la deuxième et de la troisième phrases de la règle 6.4.a).

**Cadre II Observations - lorsqu'il y a absence d'unité de l'invention (suite du point 2 de la première feuille)**

L'administration chargée de la recherche internationale a trouvé plusieurs inventions dans la demande internationale, à savoir:

1. ☐ Comme toutes les taxes additionnelles ont été payées dans les délais par le déposant, le présent rapport de recherche internationale porte sur toutes les revendications pouvant faire l'objet d'une recherche.
2. ☐ Comme toutes les recherches portant sur les revendications qui s'y prêtaient ont pu être effectuées sans effort particulier justifiant une taxe additionnelle, l'administration n'a sollicité le paiement d'aucune taxe de cette nature.
3. ☐ Comme une partie seulement des taxes additionnelles demandées a été payée dans les délais par le déposant, le présent rapport de recherche internationale ne porte que sur les revendications pour lesquelles les taxes ont été payées, à savoir les revendications n<sup>os</sup>
4. ☐ Aucune taxe additionnelle demandée n'a été payée dans les délais par le déposant. En conséquence, le présent rapport de recherche internationale ne porte que sur l'invention mentionnée en premier lieu dans les revendications; elle est couverte par les revendications n<sup>os</sup>

Remarque quant à la réserve

- ☐ Les taxes additionnelles étaient accompagnées d'une réserve de la part du déposant.
- ☐ Le paiement des taxes additionnelles n'était assorti d'aucune réserve.



A. CLASSEMENT DE L'OBJET DE LA DEMANDE

CIB 6 C12N15/12 C07K14/47 C07K16/18 G01N33/574 C12Q1/68  
A61K38/17 A61K48/00 A61K39/395

Selon la classification internationale des brevets (CIB) ou à la fois selon la classification nationale et la CIB

B. DOMAINES SUR LESQUELS LA RECHERCHE A PORTE

Documentation minimale consultée (système de classification suivi des symboles de classement)

CIB 6 C12N C07K G01N C12Q A61K

Documentation consultée autre que la documentation minimale dans la mesure où ces documents relèvent des domaines sur lesquels a porté la recherche

Base de données électronique consultée au cours de la recherche internationale (nom de la base de données, et si cela est réalisable, termes de recherche utilisés)

C. DOCUMENTS CONSIDERES COMME PERTINENTS

Catégorie °	Identification des documents cités, avec, le cas échéant, l'indication des passages pertinents	no. des revendications visées
X	JÉRÔME HONNORAT ET AL.: "Antibodies to a subpopulation of glial cells and a 66kDa developmental protein in patients with paraneoplastic neurological syndromes" JOURNAL OF NEUROLOGY, NEUROSURGERY AND PSYCHIATRY, vol. 61, 1996, pages 270-278, XP002045119 cité dans la demande voir page 270, colonne de gauche, alinéa 2 voir page 272, colonne de droite, alinéa 2 voir page 276, colonne de droite, alinéa 2 voir page 277, colonne de droite, alinéa 1 --- -/--	1,2,7-18

☒ Voir la suite du cadre C pour la fin de la liste des documents

☐ Les documents de familles de brevets sont indiqués en annexe

° Catégories spéciales de documents cités:

"A" document définissant l'état général de la technique, non considéré comme particulièrement pertinent

"E" document antérieur, mais publié à la date de dépôt international ou après cette date

"L" document pouvant jeter un doute sur une revendication de priorité ou cité pour déterminer la date de publication d'une autre citation ou pour une raison spéciale (telle qu'indiquée)

"O" document se référant à une divulgation orale, à un usage, à une exposition ou tous autres moyens

"P" document publié avant la date de dépôt international, mais postérieurement à la date de priorité revendiquée

"T" document ultérieur publié après la date de dépôt international ou la date de priorité et n'appartenant pas à l'état de la technique pertinent, mais cité pour comprendre le principe ou la théorie constituant la base de l'invention

"X" document particulièrement pertinent; l'invention revendiquée ne peut être considérée comme nouvelle ou comme impliquant une activité inventive par rapport au document considéré isolément

"Y" document particulièrement pertinent; l'invention revendiquée ne peut être considérée comme impliquant une activité inventive lorsque le document est associé à un ou plusieurs autres documents de même nature, cette combinaison étant évidente pour une personne du métier

"Z" document qui fait partie de la même famille de brevets

Date à laquelle la recherche internationale a été effectivement achevée

30 juin 1998

Date d'expédition du présent rapport de recherche internationale

23.07.98

Nom et adresse postale de l'administration chargée de la recherche internationale

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Fonctionnaire autorisé

Montero Lopez, B



C.(suite) DOCUMENTS CONSIDERES COMME PERTINENTS

Catégorie	Identification des documents cités, avec, le cas échéant, l'indication des passages pertinents	no. des revendications visées
X	<p>TAMARA BYK ET AL.: "Identification and molecular characterization of Unc-33-like phosphoprotein (Ulip), a putative mammalian homolog of the axonal guidance-associated unc-33 gene product" JOURNAL OF NEUROSCIENCE, vol. 16, no. 2, 15 janvier 1996, pages 688-701, XP002045120 cité dans la demande voir abrégé voir page 690, colonne de gauche, alinéa 8 - page 693, colonne de gauche, alinéa 2 voir page 693, colonne de droite, alinéa 3 - page 694, colonne de gauche, alinéa 1 voir page 697, colonne de droite, alinéa 2 - page 701, colonne de droite, dernier alinéa</p>	1,3,5-7, 10,11, 15,17,18
X	<p>YOSHIO GOSHIMA ET AL.: "Collapsin-induced growth cone collapse mediated by an intracellular protein related to UNC-33" NATURE, vol. 376, no. 6540, 10 août 1995, LONDON GB, pages 509-514, XP002069770 cité dans la demande voir page 509, colonne de droite, alinéa 1 - page 514, colonne de gauche, dernier alinéa; figure 1C</p>	1,3-7
X	<p>Base de données EMBL Numéro d'accès Q62950; 1 Novembre 1996 WANG, L. ET AL. XP002045123 voir le document en entier</p>	1
A	<p>NAOKI HAMAJIMA ET AL.: "A novel gene family defined by human dihydropyrimidinase and three related proteins with differential tissue distribution" GENE, vol. 180, no. 1/2, 1996, AMSTERDAM NL, pages 157-163, XP002045121 voir le document en entier</p>	1-7
A	<p>Base de données EMBL entrée HS901245 Numéro d'accès H85901; 22 Novembre 1995 HILLIER L. ET AL.: "The WashU-Merck EST Project." XP002045124 voir le document en entier</p>	3,4

-/--



C.(suite) DOCUMENTS CONSIDERES COMME PERTINENTS		
Catégorie	Identification des documents cités, avec, le cas échéant, l'indication des passages pertinents	no. des revendications visées
A	<p>BYK T ET AL: "The Ulips: A growing protein family related to the axonal guidance associated UNC-33 protein."  26TH ANNUAL MEETING OF THE SOCIETY FOR NEUROSCIENCE, WASHINGTON, D.C., USA, NOVEMBER 16-21, 1996. SOCIETY FOR NEUROSCIENCE ABSTRACTS 22 (1-3). 1996. 1713. ISSN: 0190-5295, XP002045122  * abrégé 676.9 *</p> <p>---</p>	1-18
A	<p>JANE E. MINTURN ET AL.: "TOAD-64, a gene expressed early in neuronal differentiation in the rat, is related to unc-33, a C. elegans gene involved in axon outgrowth"  THE JOURNAL OF NEUROSCIENCE, vol. 15, no. 10, octobre 1995, pages 6757-6766, XP002069771  voir le document en entier</p> <p>---</p>	1-18
P,X	<p>Base de données Trrod  Numéro d'accès 008553; 1 Juillet 1997  BYK T. ET AL.: "ULIP2 Protein"  XP002069773  voir le document en entier</p> <p>---</p>	1,7,10, 15
P,X	<p>Base de données Trrod  Numéro d'accès 008554; 1 Juillet 1997  BYK T. ET AL.: "ULIP3 Protein"  XP002069774  voir le document en entier</p> <p>---</p>	1,7,10, 15
P,X	<p>Base de données Trrod  Numéro d'accès 008886; 1 Juillet 1997  BYK T. ET AL.: "ULIP4 Protein"  XP002069775  voir le document en entier</p> <p>---</p>	1,7,10, 15
P,X	<p>GAETANO, CARLO ET AL: "Identification and characterization of a retinoic acid-regulated human homolog of the unc - 33 - like phosphoprotein gene (hUlip) from neuroblastoma cells"  J. BIOL. CHEM. (1997), 272(18), 12195-12201 CODEN: JBCHA3; ISSN: 0021-9258, 1997, XP002069772  voir page 12195, colonne de droite, alinéa 2  voir page 12196, colonne de droite, alinéa 2 - page 12201, colonne de gauche, alinéa 2</p> <p>-----</p>	1-18



11-11-11



# RAPPORT DE RECHERCHE INTERNATIONALE

Demande internationale No

PCT/FR 98/00328

## A. CLASSEMENT DE L'OBJET DE LA DEMANDE

CIB 6 C12N15/12 C07K14/47 C07K16/18 G01N33/574 C12Q1/68  
A61K38/17 A61K48/00 A61K39/395

Selon la classification internationale des brevets (CIB) ou à la fois selon la classification nationale et la CIB

## B. DOMAINES SUR LESQUELS LA RECHERCHE A PORTE

Documentation minimale consultée (système de classification suivi des symboles de classement)

CIB 6 C12N C07K G01N C12Q A61K

Documentation consultée autre que la documentation minimale dans la mesure où ces documents relèvent des domaines sur lesquels a porté la recherche

Base de données électronique consultée au cours de la recherche internationale (nom de la base de données, et si cela est réalisable, termes de recherche utilisés)

## C. DOCUMENTS CONSIDERES COMME PERTINENTS

Catégorie *	Identification des documents cités, avec, le cas échéant, l'indication des passages pertinents	no. des revendications visées
X	JÉRÔME HONNORAT ET AL.: "Antibodies to a subpopulation of glial cells and a 66kDa developmental protein in patients with paraneoplastic neurological syndromes" JOURNAL OF NEUROLOGY, NEUROSURGERY AND PSYCHIATRY, vol. 61, 1996, pages 270-278, XP002045119 cité dans la demande voir page 270, colonne de gauche, alinéa 2 voir page 272, colonne de droite, alinéa 2 voir page 276, colonne de droite, alinéa 2 voir page 277, colonne de droite, alinéa 1 --- -/-	1,2,7-18

☒ Voir la suite du cadre C pour la fin de la liste des documents

☐ Les documents de familles de brevets sont indiqués en annexe

### \* Catégories spéciales de documents cités:

"A" document définissant l'état général de la technique, non considéré comme particulièrement pertinent

"E" document antérieur, mais publié à la date de dépôt international ou après cette date

"L" document pouvant jeter un doute sur une revendication de priorité ou cité pour déterminer la date de publication d'une autre citation ou pour une raison spéciale (telle qu'indiquée)

"O" document se référant à une divulgation orale, à un usage, à une exposition ou tous autres moyens

"P" document publié avant la date de dépôt international, mais postérieurement à la date de priorité revendiquée

"T" document ultérieur publié après la date de dépôt international ou la date de priorité et n'appartenant pas à l'état de la technique pertinent, mais cité pour comprendre le principe ou la théorie constituant la base de l'invention

"X" document particulièrement pertinent; l'invention revendiquée ne peut être considérée comme nouvelle ou comme impliquant une activité inventive par rapport au document considéré isolément

"Y" document particulièrement pertinent; l'invention revendiquée ne peut être considérée comme impliquant une activité inventive lorsque le document est associé à un ou plusieurs autres documents de même nature, cette combinaison étant évidente pour une personne du métier

"Z" document qui fait partie de la même famille de brevets

Date à laquelle la recherche internationale a été effectivement achevée

30 juin 1998

Date d'expédition du présent rapport de recherche internationale

23.07.98

Nom et adresse postale de l'administration chargée de la recherche internationale

Office Européen des Brevets, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Fonctionnaire autorisé

Montero Lopez, B

# RAPPORT DE RECHERCHE INTERNATIONALE

Formi Internationale No

PCT/FR 98/00328

## C.(suite) DOCUMENTS CONSIDERES COMME PERTINENTS

Catégorie	Identification des documents cités, avec, le cas échéant, l'indication des passages pertinents	no. des revendications visées
X	<p>TAMARA BYK ET AL.: "Identification and molecular characterization of Unc-33-like phosphoprotein (Ulip), a putative mammalian homolog of the axonal guidance-associated unc-33 gene product" JOURNAL OF NEUROSCIENCE, vol. 16, no. 2, 15 janvier 1996, pages 688-701, XP002045120 cité dans la demande voir abrégé voir page 690, colonne de gauche, alinéa 8 - page 693, colonne de gauche, alinéa 2 voir page 693, colonne de droite, alinéa 3 - page 694, colonne de gauche, alinéa 1 voir page 697, colonne de droite, alinéa 2 - page 701, colonne de droite, dernier alinéa</p> <p>---</p>	1,3,5-7, 10,11, 15,17,18
X	<p>YOSHIO GOSHIMA ET AL.: "Collapsin-induced growth cone collapse mediated by an intracellular protein related to UNC-33" NATURE, vol. 376, no. 6540, 10 août 1995, LONDON GB, pages 509-514, XP002069770 cité dans la demande voir page 509, colonne de droite, alinéa 1 - page 514, colonne de gauche, dernier alinéa; figure 1C</p> <p>---</p>	1,3-7
X	<p>Base de données EMBL Numéro d'accès Q62950; 1 Novembre 1996 WANG, L. ET AL. XP002045123 voir le document en entier</p> <p>---</p>	1
A	<p>NAOKI HAMAJIMA ET AL.: "A novel gene family defined by human dihydropyrimidinase and three related proteins with differential tissue distribution" GENE, vol. 180, no. 1/2, 1996, AMSTERDAM NL, pages 157-163, XP002045121 voir le document en entier</p> <p>---</p>	1-7
A	<p>Base de données EMBL entrée HS901245 Numéro d'accès H85901; 22 Novembre 1995 HILLIER L. ET AL.: "The WashU-Merck EST Project." XP002045124 voir le document en entier</p> <p>---</p>	3,4
	<p>---</p> <p>-/--</p>	

# RAPPORT DE RECHERCHE INTERNATIONALE

Département internationale No

PCT/FR 98/00328

## C.(suite) DOCUMENTS CONSIDERES COMME PERTINENTS

Catégorie	Identification des documents cités, avec, le cas échéant, l'indication des passages pertinents	no. des revendications visées
A	<p>BYK T ET AL: "The Ulips: A growing protein family related to the axonal guidance associated UNC-33 protein." 26TH ANNUAL MEETING OF THE SOCIETY FOR NEUROSCIENCE, WASHINGTON, D.C., USA, NOVEMBER 16-21, 1996. SOCIETY FOR NEUROSCIENCE ABSTRACTS 22 (1-3). 1996. 1713. ISSN: 0190-5295, XP002045122 * abrégé 676.9 *</p>	1-18
A	<p>--- JANE E. MINTURN ET AL.: "TOAD-64, a gene expressed early in neuronal differentiation in the rat, is related to unc-33, a C. elegans gene involved in axon outgrowth" THE JOURNAL OF NEUROSCIENCE, vol. 15, no. 10, octobre 1995, pages 6757-6766, XP002069771 voir le document en entier</p>	1-18
P,X	<p>--- Base de données Trrod Numéro d'accès 008553; 1 Juillet 1997 BYK T. ET AL.: "ULIP2 Protein" XP002069773 voir le document en entier</p>	1,7,10, 15
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P,X	<p>--- Base de données Trrod Numéro d'accès 008886; 1 Juillet 1997 BYK T. ET AL.: "ULIP4 Protein" XP002069775 voir le document en entier</p>	1,7,10, 15
P,X	<p>--- GAETANO, CARLO ET AL: "Identification and characterization of a retinoic acid-regulated human homolog of the unc - 33 - like phosphoprotein gene (hulip) from neuroblastoma cells" J. BIOL. CHEM. (1997), 272(18), 12195-12201 CODEN: JBCHA3; ISSN: 0021-9258, 1997, XP002069772 voir page 12195, colonne de droite, alinéa 2 voir page 12196, colonne de droite, alinéa 2 - page 12201, colonne de gauche, alinéa 2</p>	1-18

# RAPPORT DE RECHERCHE INTERNATIONALE

D. .nde internationale n°  
PCT/FR 98/00328

## **Cadre I Observations - lorsqu'il a été estimé que certaines revendications ne pouvaient pas faire l'objet d'une recherche (suite du point 1 de la première feuille)**

Conformément à l'article 17.2)a), certaines revendications n'ont pas fait l'objet d'une recherche pour les motifs suivants:

1. ☒ Les revendications n<sup>os</sup> se rapportent à un objet à l'égard duquel l'administration n'est pas tenue de procéder à la recherche, à savoir:  

Bien que la revendication 18 concerne une méthode de traitement du corps humain/animal, la recherche a été effectuée et basée sur les effets imputés aux produits.
2. ☐ Les revendications n<sup>os</sup> se rapportent à des parties de la demande internationale qui ne remplissent pas suffisamment les conditions prescrites pour qu'une recherche significative puisse être effectuée, en particulier:
3. ☐ Les revendications n<sup>os</sup> sont des revendications dépendantes et ne sont pas rédigées conformément aux dispositions de la deuxième et de la troisième phrases de la règle 6.4.a).

## **Cadre II Observations - lorsqu'il y a absence d'unité de l'invention (suite du point 2 de la première feuille)**

L'administration chargée de la recherche internationale a trouvé plusieurs inventions dans la demande internationale, à savoir:

1. ☐ Comme toutes les taxes additionnelles ont été payées dans les délais par le déposant, le présent rapport de recherche internationale porte sur toutes les revendications pouvant faire l'objet d'une recherche.
2. ☐ Comme toutes les recherches portant sur les revendications qui s'y prêtaient ont pu être effectuées sans effort particulier justifiant une taxe additionnelle, l'administration n'a sollicité le paiement d'aucune taxe de cette nature.
3. ☐ Comme une partie seulement des taxes additionnelles demandées a été payée dans les délais par le déposant, le présent rapport de recherche internationale ne porte que sur les revendications pour lesquelles les taxes ont été payées, à savoir les revendications n<sup>os</sup>
4. ☐ Aucune taxe additionnelle demandée n'a été payée dans les délais par le déposant. En conséquence, le présent rapport de recherche internationale ne porte que sur l'invention mentionnée en premier lieu dans les revendications; elle est couverte par les revendications n<sup>os</sup>

Remarque quant à la réserve

- ☐ Les taxes additionnelles étaient accompagnées d'une réserve de la part du déposant.
- ☐ Le paiement des taxes additionnelles n'était assorti d'aucune réserve.

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PATENT COOPERATION TREATY

# PCT

## INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference BET 98/0077	<b>FOR FURTHER ACTION</b> See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/FR98/00328	International filing date (day/month/year) 19 February 1998 (19.02.1998)	Priority date (day/month/year) 19 February 1997 (19.02.1997)
International Patent Classification (IPC) or national classification and IPC C12N 15/12		
Applicant INSTITUT NATIONAL DE LA SANTE ET DE LA RECHERCHE MEDICALE (INSERM)		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

☒ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of 4 sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☒ Certain observations on the international application

Date of submission of the demand 06 August 1998 (06.08.1998)	Date of completion of this report 26 May 1999 (26.05.1999)
Name and mailing address of the IPEA/EP European Patent Office D-80298 Munich, Germany Facsimile No. 49-89-2399-4465	Authorized officer  Telephone No. 49-89-2399-0



# INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/FR98/00328

## I. Basis of the report

1. This report has been drawn on the basis of *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.)*:

- ☐ the international application as originally filed.
- ☒ the description, pages 1-69, as originally filed,  
 pages \_\_\_\_\_, filed with the demand,  
 pages \_\_\_\_\_, filed with the letter of \_\_\_\_\_,  
 pages \_\_\_\_\_, filed with the letter of \_\_\_\_\_.
- ☒ the claims, Nos. \_\_\_\_\_, as originally filed,  
 Nos. \_\_\_\_\_, as amended under Article 19,  
 Nos. \_\_\_\_\_, filed with the demand,  
 Nos. 1-19, filed with the letter of 21 April 1999 (21.04.1999),  
 Nos. \_\_\_\_\_, filed with the letter of \_\_\_\_\_.
- ☒ the drawings, sheets/fig 1/24-24/24, as originally filed,  
 sheets/fig \_\_\_\_\_, filed with the demand,  
 sheets/fig \_\_\_\_\_, filed with the letter of \_\_\_\_\_,  
 sheets/fig \_\_\_\_\_, filed with the letter of \_\_\_\_\_.

2. The amendments have resulted in the cancellation of:

- ☐ the description, pages \_\_\_\_\_
- ☐ the claims, Nos. \_\_\_\_\_
- ☐ the drawings, sheets/fig \_\_\_\_\_

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).

4. Additional observations, if necessary:





**V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement****1. Statement**

Novelty (N)	Claims	1-7, 9, 10, 13-19	YES
	Claims	8, 11, 12	NO
Inventive step (IS)	Claims	1-7, 9, 10, 13-15, 17	YES
	Claims	8, 11, 12, 16, 18, 19	NO
Industrial applicability (IA)	Claims	1-19	YES
	Claims		NO

**2. Citations and explanations**

1. In the drawing up of this report, the priority documents were not available to the examiner. The report has therefore been drawn up on the assumption that the right to priority is valid.
2. The application discloses a family of proteins called ULIP. The presence of antibodies recognizing these proteins appears to be correlated with certain paraneoplastic neurological syndromes and certain cancers. The claims relate to the ULIP proteins and to the nucleotide sequences coding them, to antibodies recognizing these proteins and to various uses.

**3.1 Novelty (PCT Article 33(2))**

The polypeptides of Seq. ID Nos. 2, 4, 6 and 8 as claimed, together with the nucleic acid sequences coding for said polypeptides, have not been described in the prior art. Consequently, Claims 1 to 7, 9 and 10 are novel.

Antibodies detecting the protein POP-66, and which can be used for diagnosing paraneoplastic



neurological syndromes, have been described in Honnorat et al., 1996 (cited in the international search report). These antibodies appear to have been "obtained" from the protein POP-66. Claim 8 and, since the protein POP-66 forms part of the ULIP family, Claims 11 and 12 are therefore considered to lack novelty.

The uses described in Claims 14 to 19 have not been described in the prior art and are therefore novel.

### 3.2 Inventive step (PCT Article 33(3))

Honnorat et al., 1996 (cited in the international search report), have described antibodies isolated from the serum of patients with a paraneoplastic neurological syndrome. These antibodies are capable of recognizing an antigen combined with particular brain cells. The present application differs from this document by the disclosure of the isolation and cloning of the protein recognized by said antibodies. The technical problem to be solved by the application can therefore be formulated as that of isolating this protein. Since direct cloning from a DNA bank of the human brain was not possible (see p.22, lines 21 to 24), a person skilled in the art would not have arrived at the claimed sequences in an obvious manner. Claims 1 to 7, 9, 10, 14, 15 and 17 are therefore considered to be inventive.

Several proteins from the family of proteins called ULIP and their role in the development of the nervous system have been described (see the documents cited in the international search report). Their use in the treatment of neurological



INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/FR 98/00328

infections therefore appears to have been obvious to a person skilled in the art. Consequently, Claims 16, 18 and 19 are considered not to involve an inventive step.



INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.  
PCT/FR 98/00328

**VIII. Certain observations on the international application**

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

- 4.1 The scope of Claim 10 is unclear. In the absence of defined functions, the phrase "biologically active" has no technical meaning.





# TRAITE DE COOPERATION EN MATIERE DE BREVETS

## PCT

REC'D 28 MAY 1999

WIPO PCT

### RAPPORT D'EXAMEN PRELIMINAIRE INTERNATIONAL

(article 36 et règle 70 du PCT)

41



Référence du dossier du déposant ou du mandataire BET 98/0077	<b>POUR SUITE A DONNER</b> voir la notification de transmission du rapport d'examen préliminaire international (formulaire PCT/IPEA/416)	
Demande internationale n° PCT/FR98/00328	Date du dépôt international (jour/mois/année) 19/02/1998	Date de priorité (jour/mois/année) 19/02/1997
Classification internationale des brevets (CIB) ou à la fois classification nationale et CIB C12N15/12		
Déposant INSTITUT NATIONAL DE LA SANTE ET DE ... et al.		

- Le présent rapport d'examen préliminaire international, établi par l'administration chargée de l'examen préliminaire international, est transmis au déposant conformément à l'article 36.
- Ce RAPPORT comprend 5 feuilles, y compris la présente feuille de couverture.
  - ☒ Il est accompagné d'ANNEXES, c'est-à-dire de feuilles de la description, des revendications ou des dessins qui ont été modifiées et qui servent de base au présent rapport ou de feuilles contenant des rectifications faites auprès de l'administration chargée de l'examen préliminaire international (voir la règle 70.16 et l'instruction 607 des Instructions administratives du PCT).

Ces annexes comprennent 4 feuilles.

- Le présent rapport contient des indications relatives aux points suivants:

- I ☒ Base du rapport
- II ☐ Priorité
- III ☐ Absence de formulation d'opinion quant à la nouveauté, l'activité inventive et la possibilité d'application industrielle
- IV ☐ Absence d'unité de l'invention
- V ☒ Déclaration motivée selon l'article 35(2) quant à la nouveauté, l'activité inventive et la possibilité d'application industrielle; citations et explications à l'appui de cette déclaration
- VI ☐ Certains documents cités
- VII ☐ Irrégularités dans la demande internationale
- VIII ☒ Observations relatives à la demande internationale

Date de présentation de la demande d'examen préliminaire internationale 06/08/1998	Date d'achèvement du présent rapport 26.05.99
Nom et adresse postale de l'administration chargée de l'examen préliminaire international:  Office européen des brevets D-80298 Munich Tél. (+49-89) 2399-0 Tx: 523656 epmu d Fax: (+49-89) 2399-4465	Fonctionnaire autorisé Stolz, B N° de téléphone (+49-89) 2399 8416 



**RAPPORT D'EXAMEN  
PRELIMINAIRE INTERNATIONAL**

Demande internationale n° PCT/FR98/00328

**I. Base du rapport**

1. Ce rapport a été rédigé sur la base des éléments ci-après *(les feuilles de remplacement qui ont été remises à l'office récepteur en réponse à une invitation faite conformément à l'article 14 sont considérées, dans le présent rapport, comme "initialement déposées" et ne sont pas jointes en annexe au rapport puisqu'elles ne contiennent pas de modifications.)* :

**Description, pages:**

1-69                      version initiale

**Revendications, N°:**

1-19                      reçue(s) le                      21/04/1999    avec lettre du                      21/04/1999

**Dessins, feuilles:**

1/24-24/24                      version initiale

2. Les modifications ont entraîné l'annulation :

- ☐ de la description,    pages :  
☐ des revendications, n°s :  
☐ des dessins,            feuilles :

3. ☐ Le présent rapport a été formulé abstraction faite (de certaines) des modifications, qui ont été considérées comme allant au-delà de l'exposé de l'invention tel qu'il a été déposé, comme il est indiqué ci-après (règle 70.2(c)) :

4. Observations complémentaires, le cas échéant :



**RAPPORT D'EXAMEN  
PRELIMINAIRE INTERNATIONAL**

Demande internationale n° PCT/FR98/00328

**V. Déclaration motivée selon l'article 35(2) quant à la nouveauté, l'activité inventive et la possibilité d'application industrielle; citations et explications à l'appui de cette déclaration**

**1. Déclaration**

Nouveauté	Oui : Revendications 1-7, 9, 10, 13-19
	Non : Revendications 8, 11, 12
Activité inventive	Oui : Revendications 1-7, 9, 10, 13-15, 17
	Non : Revendications 8, 11, 12, 16, 18, 19
Possibilité d'application industrielle	Oui : Revendications 1-19
	Non : Revendications

**2. Citations et explications**

**voir feuille séparée**

**VIII. Observations relatives à la demande internationale**

Les observations suivantes sont faites au sujet de la clarté des revendications, de la description et des dessins et de la question de savoir si les revendications se fondent entièrement sur la description :

**voir feuille séparée**



1. Lors de l'établissement de ce rapport, les documents de priorité n'étaient pas à la disposition de l'examineur. Ce rapport a été établi en supposant un droit de priorité valable.
2. La demande divulgue une famille de protéines, dénommées ULIP. La présence d'anticorps reconnaissant ces protéines semble être corrélée avec certains syndromes neurologiques paranéoplasiques et certains cancers. Les revendications se rapportent aux protéines ULIP et aux séquences nucléotidiques codant celles-ci, à des anticorps reconnaissant ces protéines et à des utilisations diverses.

3. Déclaration motivée

3.1. Nouveauté (art. 33(2) PCT)

Les polypeptides de Seq. ID nos. 2, 4, 6, et 8 tels que revendiqués ainsi que les séquences d'acides nucléiques codant pour lesdits polypeptides n'ont pas été décrits dans l'art antérieur. Par conséquent, les revendications 1 à 7, 9 et 10 sont nouvelles.

Des anticorps détectant la protéine POP-66, et utilisables pour le diagnostic de syndromes neurologiques paranéoplastiques ont été décrits dans Honnorat et al., 1996 (cité dans le RRI). Il semble que ces anticorps soient "obtenus" à partir de la protéine POP-66. La revendication 8, et parce que la protéine POP-66 fait partie de la famille ULIP, les revendications 11 et 12 sont donc considérées comme manquant de nouveauté.

Les utilisations selon les revendications 14 à 19 n'ont pas été décrites dans l'art antérieur et sont donc nouvelles.

3.2. Activité inventive (art. 33(3) PCT)

Honnorat et al., 1996 (cité dans le RRI) ont décrit des anticorps isolés du sérum de patients avec un syndrome neurologique paranéoplasique. Ces anticorps sont capables de reconnaître un antigène associé avec des cellules particulières du





cerveau. La présente demande se distingue de ce document par la divulgation de l'isolement et le clonage de la protéine reconnue par lesdits anticorps. Le problème technique que se propose de résoudre la demande peut donc être formulé comme l'isolement de cette protéine. Comme le clonage direct à partir d'une banque d'ADN de cerveau humain n'était pas possible (voir p. 22, lignes 21-24), l'homme du métier ne serait pas arrivé aux séquences revendiquées d'une manière évidente. Les revendications 1 à 7, 9, 10, 14, 15 et 17 sont donc considérées comme inventives.

Plusieurs protéines de la famille de protéines dénommées ULIP et leur rôle dans le développement du système nerveux ont été décrits (voir les documents cités dans le RRI). Il semble donc que leur utilisation dans le traitement d'affections neurologiques était évident pour l'homme du métier. Par conséquent, les revendications 16, 18 et 19 sont considérées comme manquant d'activité inventive.

#### 4. Observations relatives à la demande internationale

- 4.1. La portée de la revendication 10 n'est pas claire. En l'absence de fonctions définies, le terme "biologiquement actif" n'a aucune signification technique.



## REVENDICATIONS

1. Polypeptide purifié comprenant une séquence d'acides  
5 aminés choisie parmi SEQ ID n° 2, n° 4, n° 6 et n° 8.
2. Polypeptide purifié selon la revendication 1 comprenant  
la séquence d'acides aminés SEQ ID n° 8, ledit polypeptide étant désigné  
par « POP-66 ».
3. Acide nucléotidique isolé comprenant une séquence  
10 codant pour un polypeptide de séquence d'acides aminés SEQ ID n° 2,  
n° 4, n° 6 ou n° 8.
4. Acide nucléique selon la revendication 3, comprenant une  
séquence choisie parmi SEQ ID n° 1, n° 3, n° 5 ou n° 7, codant  
respectivement pour un polypeptide de séquence d'acides aminés SEQ  
15 ID n° 2, n° 4, n° 6 ou n° 8.
5. Acide nucléique selon la revendication 4, comprenant la  
séquence nucléotidique SEQ ID n° 7 codant pour un polypeptide selon la  
revendication 2.
6. Vecteur de clonage et/ou d'expression contenant une  
20 séquence d'acides nucléiques selon l'une des revendications 3 à 5.
7. Cellule hôte transfectée par un vecteur selon la  
revendication 6.
8. Anticorps mono- ou polyclonaux obtenus à partir d'un  
polypeptide selon l'une des revendications 1 et 2, ainsi que les fragments,  
25 les anticorps chimériques ou les immunoconjugués desdits anticorps  
mono- ou polyclonaux.



9. Composition utile pour le diagnostic des syndromes neurologiques paranéoplasiques et/ou pour le diagnostic précoce de la formation des tumeurs, caractérisée en ce qu'elle comprend un polypeptide purifié POP-66, selon la revendication 2.

5 10. Utilisation d'un polypeptide purifié POP 66 selon la revendication 2, dérivé ou fragment polypeptidique biologiquement actif de POP-66 ou d'un acide nucléique selon la revendication 5 pour détecter la présence d'anticorps anti-CV2 dans un échantillon biologique.

10 11. Utilisation d'anticorps mono- ou polyclonaux ou leurs fragments, anticorps chimériques ou immunoconjugués selon la revendication 8, pour la purification ou la détection d'une protéine ULIP correspondante dans un échantillon biologique.

15 12. Utilisation d'anticorps dirigés contre une protéine de la famille ULIP pour la mise en évidence d'une protéine ULIP dans des néoplasmes et les syndromes neurologiques paranéoplasiques, à des fins de diagnostic.

13. Utilisation selon la revendication 12, les anticorps étant des anticorps monoclonaux obtenus à partir du sérum polyclonal anti-CV2 de patients.

20 14. Méthode pour le diagnostic des syndromes neurologiques paranéoplasiques et/ou pour le diagnostic précoce de la formation des tumeurs cancéreuses, caractérisée en ce que l'on met en évidence dans un échantillon de sang prélevé chez un individu des auto-anticorps dirigés contre une protéine POP-66 par

25 - la mise en contact un échantillon de sang prélevé chez un individu avec un polypeptide purifié (POP-66) selon la revendication 2, dérivé ou fragment polypeptidique biologiquement actif de POP-66,



éventuellement fixé sur un support dans des conditions permettant la formation de complexes immunologiques spécifiques entre ledit polypeptide et les auto-anticorps éventuellement présents dans l'échantillon de sang, et

5 - la détection des complexes immunologiques spécifiques éventuellement formés.

15. Kit pour le diagnostic des syndromes neurologiques paranéoplasiques et pour le diagnostic précoce de la formation des tumeurs à partir d'un prélèvement biologique comprenant :

10 - au moins un polypeptide purifié POP-66, selon la revendication 2 dérivé ou fragment polypeptidique biologiquement actif de POP-66, éventuellement fixé sur un support,

15 - des moyens de révélation de la formation de complexes antigène/anticorps spécifiques entre un auto-anticorps anti-POP-66 et ledit polypeptide purifié POP-66, dérivé ou fragment polypeptidique et/ou des moyens de quantification de ces complexes.

20 16. Composition pharmaceutique, comprenant au moins une protéine purifiée de la famille ULIP<sup>3</sup>, fragment polypeptidique ou dérivé biologiquement actif de celle-ci, une séquence ou fragment de séquence nucléotidique codant pour ladite protéine, une séquence anti-sens capable de s'hybrider spécifiquement avec une séquence nucléotidique codant pour ladite protéine, ou un anticorps dirigé contre ladite protéine, associée à un véhicule pharmaceutiquement acceptable.

25 17. Composition pharmaceutique selon la revendication 15, comprenant au moins un polypeptide purifié POP-66 selon la revendication 2, fragment polypeptidique ou dérivé biologiquement actif de celui-ci, une séquence ou fragment de séquence nucléotidique codant





pour ledit polypeptide, une séquence anti-sens capable de s'hybrider spécifiquement avec une séquence nucléotidique codant pour ledit polypeptide, ou un anticorps dirigé contre ledit polypeptide, associé à un véhicule pharmaceutiquement acceptable.

- 5                   18. Utilisation d'une protéine purifiée de la famille ULIP, fragment polypeptidique ou dérivé biologiquement actif de celle-ci, une séquence ou fragment de séquence nucléotidique codant pour ladite protéine, une séquence anti-sens capable de s'hybrider spécifiquement avec une séquence nucléotidique codant pour ladite protéine, ou un  
10 anticorps dirigé contre ladite protéine, pour la fabrication d'un médicament destiné à traiter les maladies neurodégénératives et les néoplasmes.

19. Méthode de traitement des maladies neurodégénératives et des néoplasmes comprenant l'administration à un sujet nécessitant un  
15 tel traitement d'une quantité thérapeutiquement efficace d'une protéine purifiée de la famille ULIP, fragment polypeptidique ou dérivé biologiquement actif de celle-ci, une séquence ou fragment de séquence nucléotidique codant pour ladite protéine, une séquence anti-sens capable de s'hybrider spécifiquement avec une séquence nucléotidique  
20 codant pour ladite protéine, ou un anticorps dirigé contre ladite protéine, associée à un véhicule pharmaceutiquement acceptable.



XP-002069773

ID 008553 PRELIMINARY; PRT; 572 AA.  
AC 008553;  
DT 01-JUL-1997 (TREMBLREL. 04, CREATED)  
DT 01-JUL-1997 (TREMBLREL. 04, LAST SEQUENCE UPDATE)  
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RC STRAIN=ICR OUTBRED STRAIN; TISSUE=BRAIN;  
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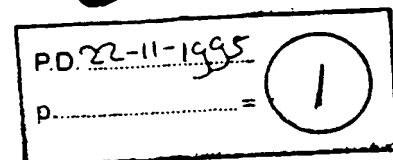
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501 GPVCEVSVTP KTVTPASSAK TSPAKQQAPP VRNLHQSGFS LSGAQIDDNI  
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XP 002045124



ID HS901245 standard; RNA; EST; 281 BP.  
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RA Trevaskis E., Waterston R., Williamson A., Wohldmann P., Wilson R.;  
RT "The WashU-Merck EST Project";  
RL Unpublished.  
XX  
CC Contact: Wilson RK WashU-Merck EST Project Washington University  
CC School of Medicine 4444 Forest Park Parkway, Box 8501, St. Louis,  
CC MO 63108 Tel: 314 286 1800 Fax: 314 286 1810 Email:  
CC est@watson.wustl.edu High quality sequence stops: 161 Source: IMAGE  
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CC ; contact the IMAGE Consortium (info@image.llnl.gov) for further  
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676.9

THE ULIPS: A GROWING PROTEIN FAMILY RELATED TO THE AXONAL GUIDANCE ASSOCIATED UNC-33 PROTEIN, T. Byk, S. Ozon, C. Cifuentes-Diaz<sup>2</sup> and A. Sobel<sup>1</sup>, INSERM U440 and <sup>2</sup>UI53, 75005 Paris - France

We have recently described a novel phosphoprotein preferentially expressed in the nervous system and strongly regulated during development. We named this protein Ulip for Unc-33 Like Phosphoprotein, based on its homology with this axonal guidance associated protein in *C. elegans*. The phosphorylation pattern of Ulip in PC12 cells is changed upon induction of neuronal differentiation with NGF. It is expressed in neurons and can also be detected in oligodendrocytes and Schwann cells.

Ulip is closely related to other unc-33 like proteins identified recently: toad-64, originally identified as a neuronal differentiation marker in the rat, crmp-62, implicated in the collapsin signal transduction in chick. Sequence analysis of these proteins as well as of human EST sequences revealed the existence of a broad Ulip protein family: Ulip1 (Ulip), Ulip2 (crmp-62 and toad-64) and Ulip3 (hcrmp-1) displaying ~75% identity between each other and more than 95% phylogenetic conservation; Ulip4 and Ulip5 displaying ~50% identity with Ulip1-Ulip3. The phylogenetic conservation of this family is emphasized by the identification of several "Ulip" sequences within the recently available *C. elegans* genome sequence.

The Ulip expression patterns during development and neuronal differentiation displayed distinct regulation. Ulip1 is strongly down-regulated in the adult brain and up-regulated in differentiated PC12 cells. Ulip2 is similarly but only weakly regulated in both cases. Ulip3 is strongly down-regulated in the adult brain, but its expression is not induced in differentiating PC12 cells. Ulip4 is down-regulated in the adult brain, but also down-regulated in differentiated PC12 cells. Finally, Ulip2-4 appeared more brain-specific than Ulip1, which was also detected in newborn heart and muscle.

This expression analysis suggests that each of the Ulip proteins might have its specific biological role, particularly in neuronal differentiation and outgrowth. Supported by INSERM, AFM, FEBS, ARC, FNCLCC

676.11

CLONING OF RAT POLYSIALYLTRANSFERASE cDNA AND DEVELOPMENTAL EXPRESSION OF ITS mRNA IN THE BRAIN. G.K. Wood<sup>1</sup>\*, J. J. Liang<sup>1</sup>, S. Ahmad<sup>2</sup>, R. Ouirion<sup>1</sup>, and L.K. Srivastava<sup>1</sup>.

<sup>1</sup>Douglas Hospital Research Centre, Dept. of Psychiatry, McGill Univ., Montreal, Quebec, Canada; <sup>2</sup>Biotechnology Research Institute, Montreal.

Polysialyltransferase (PST) is an enzyme that catalyzes polysialic acid (PSA) synthesis on neural cell adhesion molecule (NCAM), which is critical for neural development and plasticity. Based on the cDNA sequences of recently cloned hamster, mouse, and human PSTs (that are highly homologous to each other), we used a PCR strategy to clone the cDNA of the rat brain PST in order to devise probes for studying expression of the enzyme mRNA in this tissue during development and adulthood. By Northern blotting using an oligonucleotide probe, we observed a prominent band of ~5 Kb in the brain tissue which is consistent with recently reported hamster and mouse PST transcripts. As expected, the message is more abundant in the embryonic than the adult brain. By in situ hybridization, signals for PST were detected abundantly in E15 cortical neuroepithelium, hippocampal formation neuroepithelium, cerebellar neuroepithelium and basal telencephalon. At postnatal stages, PST mRNA was detected in many brain areas including neocortex, hippocampus, some thalamic nuclei, and cerebellum. In adult brain, expression of PST mRNA was observed in the olfactory bulb, cerebral cortex, hippocampus, and cerebellum. The results suggest that the expression of PST mRNA in the rat brain is developmentally regulated and that PST mRNA has more widespread distribution in the rat brain than expected from the known expression pattern of PSA-NCAM. (Supported by the FRSQ).





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 AC 008554;  
 DT 01-JUL-1997 (TREMBLREL. 04, CREATED)  
 DT 01-JUL-1997 (TREMBLREL. 04, LAST SEQUENCE UPDATE)  
 DT 01-JUL-1997 (TREMBLREL. 04, LAST ANNOTATION UPDATE)  
 DE ULIP3 PROTEIN.  
 GN ULIP3.  
 OS MUS MUSCULUS (MOUSE).  
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 OC EUTHERIA; RODENTIA.  
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 RC STRAIN=ICR OUTBRED STRAIN; TISSUE=BRAIN;  
 RA BYK T., OZON S., SOBEL A.;  
 RL SUBMITTED (MAR-1997) TO EMBL/GENBANK/DBJ DATA BANKS.  
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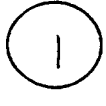
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ID 008886 PRELIMINARY; PRT; 572 AA.  
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DT 01-JUL-1997 (TREMBLREL. 04, CREATED)  
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551 ARRTAQKIMA PPGGRSNITS LS



XP-002069771

# TOAD-64, a Gene Expressed Early in Neuronal Differentiation in the Rat, Is Related to *unc-33*, a *C. elegans* Gene Involved in Axon Outgrowth

Jane E. Minturn, Hugh J. L. Fryer, Daniel H. Geschwind,\* and Susan Hockfield

Section of Neurobiology, Yale University School of Medicine, New Haven, Connecticut 06510

Using two-dimensional gel electrophoresis we previously identified membrane-associated proteins that are upregulated over the course of neurogenesis. One of these, TOAD-64 (Turned On After Division, 64 kDa), is expressed immediately after neuronal birth and is dramatically downregulated in the adult. The gene encoding TOAD-64 has now been cloned, and its sequence shows homology to the *unc-33* gene from *C. elegans*, mutations in which lead to aberrations in axon outgrowth. Northern and *in situ* hybridization show that TOAD-64 mRNA is enriched in the nervous system and is developmentally regulated in parallel with the protein. The expression of the TOAD-64 protein and gene coincident with initial neuronal differentiation and the downregulation when the majority of axon growth is complete suggests a role in axon elaboration. Three additional lines of evidence support this possibility: TOAD-64 is upregulated following neuronal induction of P19 and PC12 cells; the protein is found in lamellipodia and filopodia of growth cones; and axotomy of the sciatic nerve induces reexpression. While the sequence of TOAD-64 lacks a signal sequence and therefore is likely to encode a cytoplasmic protein, biochemical experiments demonstrate that the protein is tightly, but noncovalently, associated with membranes. The data presented here suggest that TOAD-64 could be a central element in the machinery underlying axonal outgrowth and pathfinding, perhaps playing a role in the signal transduction processes that permit growing axons to choose correct routes and targets.

**[Key words: growth cone, axonogenesis, membrane protein, axon outgrowth, regeneration, *unc-33*, differentiation, axon, development]**

As the brain develops, a population of mitotically active progenitor cells gives rise to postmitotic cells that assume the properties of neurons or glia. Postmitotic neurons elaborate processes that are involved in the migration of neurons to their adult po-

sitions as well as the elaboration of axonal and dendritic arbors. To identify proteins that participate in early functions of developing neurons, we previously used two-dimensional gel electrophoresis to compare membrane associated proteins expressed by progenitor cells to those expressed by postmitotic neurons (Geschwind and Hockfield, 1989). We compared membrane preparations from the rat neocortex at embryonic day 14 (E14), when the neocortex contains largely progenitor cells, to membrane preparations from the cortex at E21, when the neocortex contains the mature complement of neurons. Ten proteins that are upregulated greater than threefold between E14 and E21 were identified. One of these, TOAD-64 (Turned On After Division, 64 kDa, initially labeled protein 310) was selected for further study. By two-dimensional gel analysis, TOAD-64 was upregulated sevenfold between E14 and E21, appeared to be enriched in brain, and represented approximately 0.1% of total protein at E21.

Antisera raised to synthetic peptides based on amino acid sequence of TOAD-64 permitted a more detailed analysis of the regulation and distribution of TOAD-64 (Minturn et al., 1995). As predicted from the original design of the two-dimensional gel study, TOAD-64 is expressed by neurons and not by progenitor cells. It is among the earliest known proteins expressed by postmitotic neurons. The protein is dramatically downregulated during the second postnatal week to an almost undetectable level in the adult. The neural specificity and timing of expression suggested that TOAD-64 might play a role in axonogenesis or neuronal migration.

Here we report the full length sequence of a cDNA encoding TOAD-64 and show the pattern of expression of its mRNA, *in situ* and in two cell lines. TOAD-64 is homologous to the *C. elegans unc-33* gene, mutations in which lead to aberrant patterns of axon outgrowth (Hedgecock et al., 1985; Li et al., 1992). Consistent with a role in axon outgrowth in the mammalian nervous system, the TOAD-64 gene is expressed early in neuronal differentiation and is downregulated in the adult. It is reexpressed in the adult during sciatic nerve regeneration. These data, together with the regulation of expression in parallel with the neurogenesis in PC12 and P19 cells, and the localization of the TOAD-64 protein to growth cones, indicate that TOAD-64 may be a component of the intracellular machinery by which growing neurons elaborate axons.

## Materials and Methods

**PCR amplification.** Degenerate DNA oligomer primers were designed based on peptide sequences obtained from microsequencing of purified TOAD-64 protein. The only primer combination that resulted in a single major PCR product was 5'-GA(C/T)(C/T)TGT(G/T)C)AT(A/C/T)TG

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Correspondence should be addressed to Susan Hockfield, Section of Neurobiology, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06510.

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GGA/C/TCC-3' (72-fold degenerate, 5' primer) and 5'-GTC(G/A)AAI-ACIGG(A/C/G/T)C C(G/A)TC(G/A)TA-3' (32-fold degenerate, 3' primer). First strand cDNA, prepared using polyA RNA isolated from postnatal day 5 (P5) rat cortex and random primers, was used as a template for PCR. The PCR conditions were as follows: 94°C, 1 min; 49°C, 2 min; 72°C, 2 min; for 35 cycles. PCR products were analyzed by agarose gel electrophoresis, and a major band [280 base pair (bp)] was isolated and cloned into the TA cloning vector pCRII (Invitrogen). The insert was sequenced using the vector M13 and T7 primer sites. An embryonic day 18 (E18) rat brain li-

**Isolation of cDNA clones.** An embryonic day 18 (E18) rat brain library (generously provided by J. Boulter, The Salk Institute) in the vector lambda ZAP was plated on *E. coli* (strain BB4) at a density of 40,000 phage per 150 mm plate, and 400,000 clones screened according to standard techniques (Sambrook et al., 1989). Briefly, nitrocellulose filters were prehybridized at 65°C for 4–5 hr in 6× SSC (1× = 0.15 M sodium chloride, 0.015 M sodium citrate), 0.1% SDS, 1× Denhardt's (0.02% Ficoll, 0.02% BSA, 0.02% polyvinylpyrrolidone), and 100 µg/ml salmon sperm DNA. Hybridization was carried out in the same solution with the addition of 5 × 10<sup>5</sup> cpm/ml of radiolabeled probe at 65°C for 20 hr. Filters were washed once in 2× SSC, 0.1% SDS, and twice in 0.2× SSC, 0.1% SDS, at 65°C for 20 min. For the initial screening, filters were probed with the 280 bp PCR product radiolabeled ( [<sup>32</sup>P]-dCTP, Amersham) by random priming (Feinberg and Vogelstein, 1983) (NEBlot Kit, New England Biolabs, Inc.). To obtain the 5' end of the gene, an additional 200,000 clones were screened with a 230 bp region amplified from the 5'-most end of the initial clones.

**DNA sequencing and analysis.** DNA sequence was obtained by the dideoxy chain termination method using the Sequenase kit (Sanger et al., 1977) (United States Biochemical Corp.). Bluescript SK/T7 primers or cDNA specific 20-mers were used. Sequence was verified from overlapping clones or by sequencing both strands of DNA. Sequence compressions were resolved using dITP nucleotides and terminal deoxynucleotidyl transferase in the sequence reaction mix. Sequence alignments and analyses were performed using the University of Wisconsin Genetics Computer Group (GCG) software.

**Northern analysis.** Total RNA (25 µg) was denatured in 2.2 M formaldehyde, 50% formamide, 1× MOPS (3-(N-morpholino) propanesulfonic acid) buffer at 65°C for 15 min. The RNA was electrophoresed on a 1.0% agarose-formaldehyde gel with 1× MOPS buffer at 50 V with buffer recirculation, and blotted to Zetabond (BioRad). Blots were prehybridized at 65°C for 30 min in 7% SDS, 1% BSA, 0.5 M phosphate buffer (pH 6.8) (PB), and 1 mM EDTA. Hybridization was carried out overnight in the same buffer containing  $1-5 \times 10^6$  cpm/ml [ $^{32}$ P]dCTP-labeled cDNA. Blots were washed twice in 5% SDS, 0.5% BSA, 40 mM PB, 1 mM EDTA, and once in 1% SDS, 40 mM PB, 1 mM EDTA at 65°C for 20 min (Church and Gilbert, 1984) and exposed to film (Hyperfilm, Amersham) at -70°C. The ubiquitously expressed, non-developmentally regulated gene cyclophilin (Lenoir et al., 1986; Danielson, 1988) was used to determine equal loading of lanes.

**In situ hybridization.** *In situ* hybridization was carried out as described by Jaworski et al. (1994). Briefly, 12–14  $\mu$ m thick fresh-frozen sections were thaw mounted onto gelatin-coated slides and postfixated in 0.1 M phosphate-buffered 4% paraformaldehyde. Sections were probed with [<sup>32</sup>S]CTP (New England Nuclear)-labeled antisense or sense cRNA transcribed *in vitro* from a 280 bp PCR product inserted into the vector pCRII (Invitrogen). Following hybridization and washing, initial localization of probe was determined by exposing the slides to film (Hyperfilm, Amersham) for 48 hr. Autoradiograms were used as negatives for prints. For higher resolution, the slides were exposed to Kodak NTB-2 liquid emulsion, developed after 2–5 d, and counterstained with cresyl violet. An antisense probe for the gene for the middle subunit of neurofilament (NF) (Marin et al., 1992) was used as a positive control.

**Immunohistochemistry.** Sprague-Dawley rats (Charles River Laboratories) ages E17 to adult were perfused transcardially with 0.1 M sodium phosphate (pH 7.4) (PB), then 4% paraformaldehyde (PFA) in PB. Whole brains or spinal cords were dissected out and postfixed in 4% PFA for 24 hr at 4°C, then equilibrated in 30% sucrose in PB. Coronal sections of E17 brain were cut at 30  $\mu$ m, and transverse sections of adult spinal cord cut at 50  $\mu$ m on a cryostat and collected as free-floating sections in PB for immunohistochemistry. Tissue sections were incubated in rabbit anti-TOAD-64 (1:2000) (Minturn et al., 1995) at room temperature over night. The secondary antibody was HRP-conjugated goat anti-rabbit IgG (1:200, Cappel). HRP was localized using a 0.7 mg/ml solution of 3,3'-diaminobenzidine tetrahydrochloride (DAB) with 0.003%  $H_2O_2$  in PB. Sections were dehydrated in graded

ethanols, equilibrated in xylenes, and coverslipped with Permount (Fisher Scientific). Sections retrogradely labeled with Fast blue were mounted in Aqua-Poly Mount (Polysciences, Inc.).

**Cell culture.** Culture and induction of P19 cells was as previously described (McBurney et al., 1988). P19 cells (ATCC) were maintained in alpha modified MEM supplemented with 2.5% FCS and 7.5% newborn calf serum in 100 mm tissue culture plates. Cells adhering to the plates were treated with 0.05% trypsin/0.5 mM EDTA in Dulbecco's phosphate-buffered saline (DPBS; GIBCO/BRL) without  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$ , and triturated with a flame constricted pipette in complete medium, and plated into bacteriological culture dishes at a density of  $1 \times 10^5$  cell/ml. For neural induction the culture medium was supplemented with 1  $\mu\text{M}$  all *trans*-retinoic acid (day 0). Four days after induction the floating clumps of cells were treated with trypsin, dispersed by titration, and plated onto culture dishes at a density of  $5 \times 10^4$  cells/cm<sup>2</sup> in medium lacking inducer. Cells were collected and RNA prepared (as described above) at 2, 4, 6, 8, and 11 d following induction or at 4 and 8 d without induction.

PC12 cells were plated on polyornithine/laminin treated 100 mm tissue culture dishes at a density of 75 cells/mm<sup>2</sup> in Dulbecco's Modified Eagle's Medium (DMEM; GIBCO/BRL)/10% FCS. Twenty-four hours after plating the medium was replaced with medium containing 100 ng/ml NGF. Cells were harvested for either RNA or protein before NGF induction and at 1, 3, 5, and 7 d following induction.

Primary neuronal cultures were made of rat sensory ganglia. Dorsal root ganglia (DRG) dissected from E16 Sprague-Dawley rat embryos in DPBS; were collected in DMEM. Following washes in Ca/Mg-free DPBS, ganglia were treated with 0.05% trypsin in Ca/Mg-free DPBS for 30 min at 37°C. After washes in DMEM supplemented with 10% fetal calf serum (Hyclone), 2 mM glutamine and penicillin-streptomycin, cells were dissociated by trituration using a fire-polished Pasteur pipette. Cells were plated onto flame sterilized, polyornithine/laminin (Sigma) coated, round cover glasses, at a density of 90 cells/mm<sup>2</sup> in medium supplemented with 50 ng/ml 2.5S NGF (Collaborative Research). After 20 hr at 37°C and 5% CO<sub>2</sub>, 4% phosphate-buffered paraformaldehyde/0.2% glutaraldehyde was added to the cultures in a volume equal to that of the culture medium. After 10 min the cells were washed extensively with 0.1 M phosphate buffer. Cultures were incubated for 2 hr with DMEM supplemented with 5% fetal calf medium, 0.1% glycine, 0.1% lysine, and 0.2% Triton X-100.

Immunohistochemistry was performed as described above, using either rabbit antisera to TOAD-64 or an antibody to class III  $\beta$ -tubulin (Lee et al., 1990a,b; Easter et al., 1993; Minturn et al., 1995), generously provided by Dr. Anthony Frankfurter, University of Virginia. Antibody binding was visualized with species specific antibodies and DAB with 2% NiSO<sub>4</sub> for HRP in single-labeling studies, or with FITC or Texas red for double-labeling studies.

**Sciatic nerve lesion.** Adult Sprague-Dawley rats weighing 150–200 g were anesthetized with 400 mg/kg chloral hydrate, and the right sciatic nerve was exposed at the level of the midshaft femur. The nerve was crushed with forceps and injected (proximal to the crush) with 1  $\mu$ l of the retrograde tracer Fast blue (1% solution in PB). Survival times ranged from 4 hr to 2 weeks after surgery, at which time the animals were deeply anesthetized and perfused transcardially with 0.1 M PB followed by 4% paraformaldehyde in PB. The spinal cords were removed at the level of the lumbar enlargement and processed for immunohistochemistry as described above. The motor neurons of the anterior horn of the spinal cord labeled with Fast blue were visualized using epifluorescence microscopy with a UV filter.

**Tissue extraction and Western analysis.** Tissues from euthanized animals were dissected and used immediately or stored frozen at  $-70^{\circ}\text{C}$ . Fresh or frozen tissues were homogenized in a Teflon-glass Potter-Elvehjem homogenizer at a concentration of 0.2 gm (wet weight) of tissue/ml of phosphate-buffered saline (PBS) (50 mM sodium phosphate pH 7.4, 150 mM NaCl) containing a cocktail of protease inhibitors (1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 5 mM N-ethylmaleimide, 5 mM  $\epsilon$ -amino-n-caproic acid, 5  $\mu\text{g/ml}$  leupeptin, and 1  $\mu\text{g/ml}$  pepstatin A). Whole homogenate samples were taken directly from this preparation. Supernatant (S) and pellet (P) fractions were prepared from the homogenates as follows. The samples were centrifuged at  $100,000 \times g$  for 1 hr and the supernatant (S fraction) was saved. The resulting pellet was rehomogenized with PBS and again separated into pellet and supernatant fractions by centrifugation. For differential extractions this pellet (from the second centrifugation) was homogenized in either 0.1 M  $\text{Na}_2\text{CO}_3$ , pH 11, PBS, 2 M NaCl in PBS, 1% Triton X-100 in PBS, 10

[illegible]

*Figure 1.* cDNA and deduced amino acid sequences of TOAD-64. The coding sequence for TOAD-64 is 1716 bp, which encodes a 572 amino acid protein. The deduced protein contains several potential protein kinase C phosphorylation sites and a tyrosine kinase phosphorylation site (*dotted underlines*). The four peptide sequences obtained by microsequence analysis of purified TOAD-64 protein are *underlined*. These sequence data are available from the EMBL Nucleotide Database under accession number Z46882.

mm EDTA in PBS or 2 M urea in PBS. The samples were centrifuged as above, the supernatants saved, and the pellets washed with the appropriate buffer. Protease inhibitors were used in all buffers. Protein concentrations were determined by the method of Bradford (Bradford, 1976) using the Bio-Rad dye reagent kit and BSA as a standard.

Samples to be analyzed by immunoblotting were combined with gel loading buffer (20 mM Tris-Cl, pH 6.8, 3% SDS, 10% glycerol, 0.01% bromophenol blue) and S-mercaptoethanol (0.05%), boiled for 5 min and electrophoresed by SDS-PAGE minigel (10%) in 50 mM Tris base, 0.38 M glycine, 0.2% SDS (Laemmli, 1970). The proteins were electrophoretically transferred to nitrocellulose overnight at 50 mA in 25 mM Tris, 0.192 M glycine, and 20% methanol (Towbin et al., 1979). The blots were blocked in Blotto (5% nonfat dry milk in TBS) for 2 hr and incubated in primary antibody (diluted in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, 0.2% Tween 20, and 0.2% sodium azide) over night at room temperature. The blots were washed and incubated with alkaline phosphatase conjugated goat anti-rabbit IgG or goat anti-mouse IgG (Promega) for 2 hr at room temperature. Immunoreactive bands were visualized with Nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma).  
For autoradiography, the blots were probed with <sup>32</sup>P-labeled anti-*ras* antibody was used for

Densitometry of blots stained with TOAD-64 antibody was used for quantification of the relative amounts of TOAD-64 in soluble and pellet fractions. Images of immunoblots were captured with a Hitachi CCD camera and imported into an Apple Macintosh Quadra 650 computer using a Scion LG-3 frame grabbing board. Imported images were an-

alyzed with NIH Image V1.57 (Wayne Rasband, NIH). Integrated densities from each band were determined. For each extraction the percent of total (Fig. 10b) was determined by dividing the density of either the S or P bands by the sum of the integrated densities of both bands.

## Results

## Results

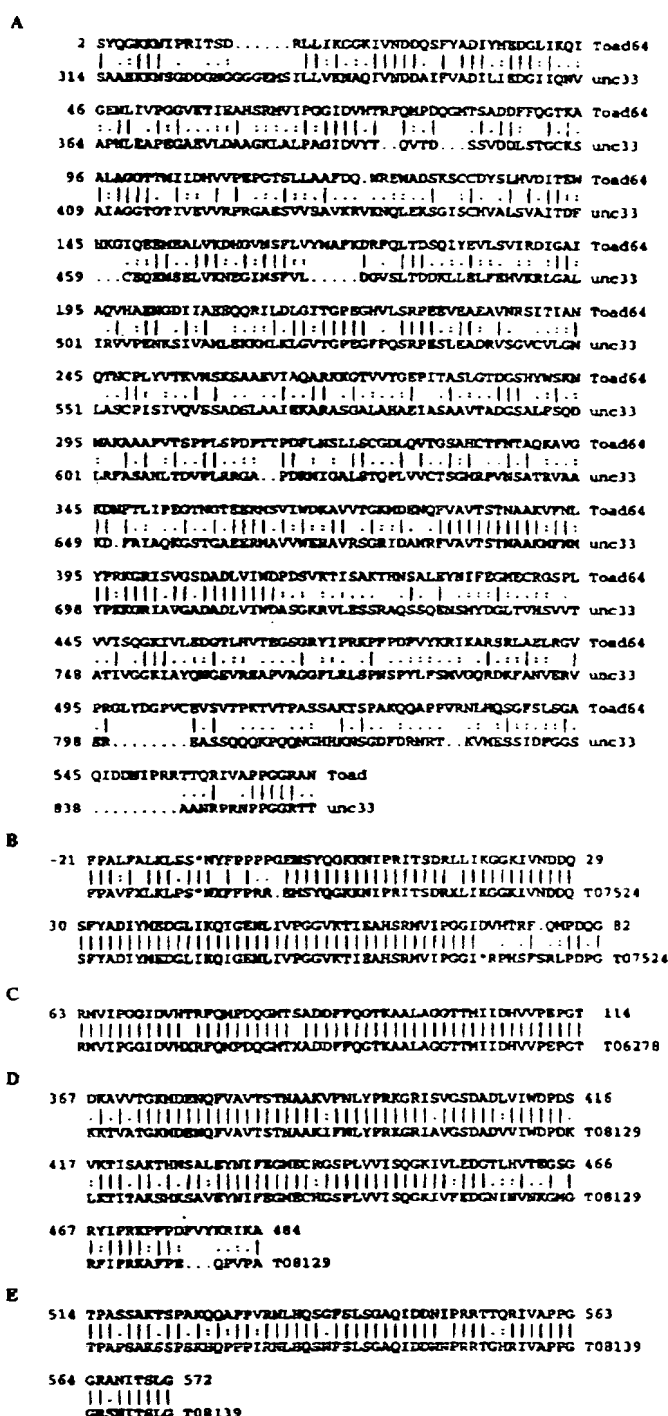
The TOAD-64 protein is expressed at its highest levels during the first postnatal week. To clone the gene encoding the TOAD-64 protein, first strand cDNA from postnatal day 5 (P5) rat cortex was used as a template for PCR amplification. Degenerate oligonucleotide primers were based on five peptide sequences obtained from microsequencing of purified TOAD-64 protein (peptide A: GAPLNSIXQG; peptide B: NHQSVAE, peptide C: YDGPVFDLTTTPK, peptide D: IAVGSDSDLVTWDPDAVK, peptide E: ALHVDIT). The primers were designed to encode only a portion of each peptide, so that a correct PCR product would be expected to contain the remainder of the peptide sequence. Of 12 possible primer combinations, one combination (representing peptide D and peptide C) amplified a single major band of approximately 280 bp, while the others amplified mul-

multiple minor bands or generated no PCR product. The major 280 bp PCR product was cloned into pCRII (Invitrogen) and sequenced. The predicted amino acid sequence from the region internal to both of the primers matched that of the original peptide fragments from the TOAD-64 protein. To obtain full length sequence, the 280 bp PCR product was used to screen an E18 rat brain lambda ZAP II cDNA library. A total of  $4.0 \times 10^5$  recombinant plaques were screened, resulting in 15 positive clones. The insert size of each of the clones was determined and redundant clones were eliminated by constructing a map of each clone using PCR with vector- and TOAD-specific primers. To obtain the 5' end of the gene, an additional 200,000 recombinants were screened with a PCR product comprising the most 5' end of the first set of clones. The composite sequence of TOAD-64 was obtained from four of these overlapping clones. The complete TOAD-64 coding sequence is 1716 bp (Fig. 1). The first in-frame methionine is preceded by a translational start consensus sequence, as described by Kozak (1984). An in-frame termination codon is found 30 bp upstream of the translation initiation site. The TOAD-64 gene encodes a 572 amino acid long protein. Analysis of the deduced amino acid sequence shows no signal sequence or likely transmembrane domain, consistent with a cytoplasmic location for the protein. The sequence also has several (S/T) X (R/K) consensus sites for potential protein kinase C phosphorylation, as well as a potential tyrosine kinase phosphorylation site, (K) XXX (D) XX (Y), at Tyr-479 (Pearson and Kemp, 1991), suggesting that TOAD-64 is a phosphoprotein.

Several converging lines of evidence indicate that the gene we have cloned encodes the TOAD-64 protein. First, the isolated gene encodes a protein with a predicted molecular mass of 62,364 daltons and an isoelectric point (pI) of 6.34. These values are remarkably similar to the molecular mass (64 kDa) and pI (6.4) of TOAD-64 (protein 310) on two-dimensional SDS-PAGE (Geschwind and Hockfield, 1989). Second, four of the original five peptides obtained by amino acid sequence analysis are represented in the cDNA sequence (see Fig. 1). And third, synthetic peptides generated from a region of the sequence (residues 381–399) that was not obtained in the original amino acid sequence analysis was used to immunize rabbits. Antisera to this new sequence recognizes TOAD-64 protein immunoprecipitated by one of the original antisera to TOAD-64 (data not shown). Together these data confirm that the isolated gene encodes the TOAD-64 protein.

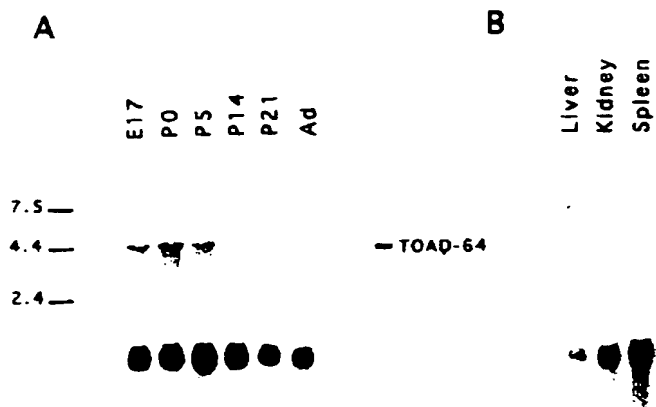
Sequence similarity analysis using the GenBank and EMBL databases at both the nucleic acid and amino acid levels indicate that TOAD-64 is a previously unreported gene. The TOAD-64 gene product is homologous to the *unc-33* gene product of *C. elegans*. Over the entire length of the proteins, TOAD-64 and *unc-33* show 34% identity, and as much as 79% similarity (Fig. 2a). *C. elegans* mutants in *unc-33* have pronounced abnormalities in axonal arborizations, presumably due to errors in axonal branching and guidance during axonogenesis (Hedgecock et al., 1985; Li et al., 1992).

Substantial amino acid homology also exists between TOAD-64 and six sequences isolated from a human fetal brain library using a random cloning strategy (Adams et al., 1993). Three of these map to overlapping stretches of the TOAD-64 gene, two map to a region in the 5' end, and one to a region in the 3' end of the gene. Over very long stretches, there is close to 100% identity between the TOAD-64 gene and the human fetal brain sequences (Fig. 2b–e). The overlap and degree of similarity be-



**Figure 2.** The predicted amino acid sequence of TOAD-64 is homologous to the *C. elegans unc-33* gene (A) and to several human fetal brain cDNAs (B–E). A, The predicted amino acid sequences for TOAD-64 and *unc-33* (Li et al., 1992) are shown aligned. Solid lines between the two sequences represent identical sequence and dots indicate conserved substitutions. The two proteins are 34% identical over the entire coding region and possess many conserved substitutions. B and C, The first 114 coding amino acids of TOAD-64 are nearly identical to human sequences T07524 (EMBL accession number) and T06278. The initiating methionine is indicated in bold. D and E, The homology between TOAD-64 and the human clones continues through the length of the protein, and ranges between 74–95% identity. The human sequence, T08129, shown in D is also found in sequences T06728 and T09404.





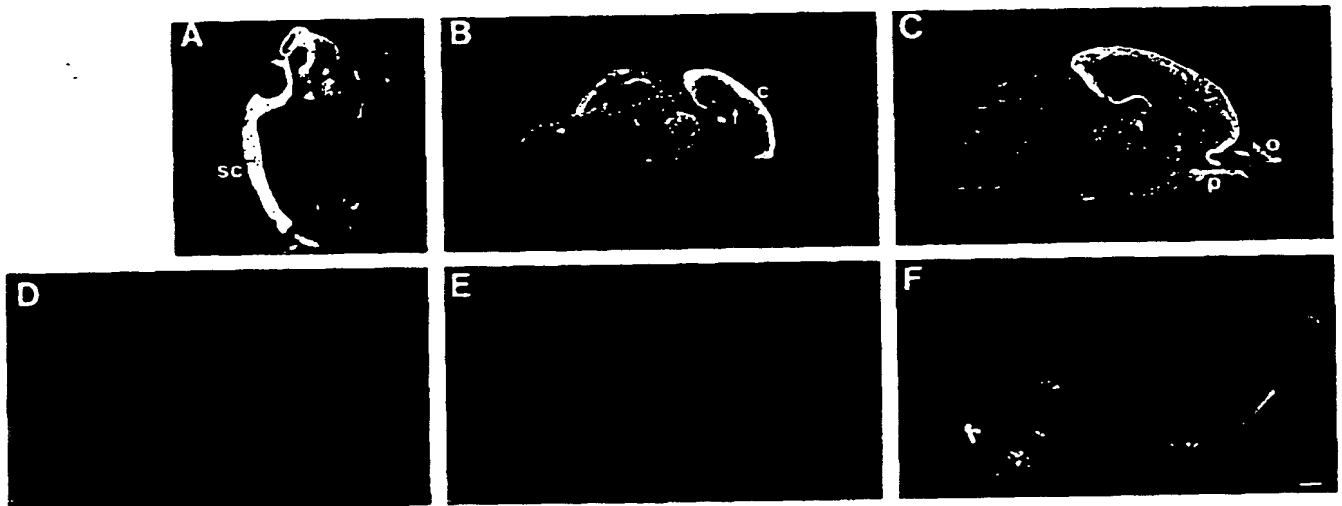
**Figure 3.** TOAD-64 mRNA is neural specific and developmentally regulated. Total RNA (25  $\mu$ g) from E17, P0, P5, P14, P21, and adult rat cortex (A) and from P21 rat liver, kidney, and spleen (B) were probed for TOAD-64 mRNA. A single 4.5 kb transcript is detected in the cortex at E17, continues to be expressed during the first postnatal week (P5), and subsequently declines during the second postnatal week to a level that is nearly undetectable in the adult. B, TOAD-64 mRNA is not detected in non-neural tissues. Even after over exposure, no TOAD-64 mRNA was detected in liver, kidney, or spleen. Both filters were simultaneously hybridized with a probe for the nondevelopmentally regulated gene, cyclophilin (*cyc*), to verify equal RNA loading levels.

tween these six gene fragments and the TOAD-64 gene suggests that all six fragments represent multiple sequences from a single gene or a gene family. These human fetal brain sequences have not been characterized, but it can be predicted that they represent the human homologue(s) of rat TOAD-64. The very high degree of conservation between rat and human could suggest that TOAD-64 has a highly conserved, essential function.

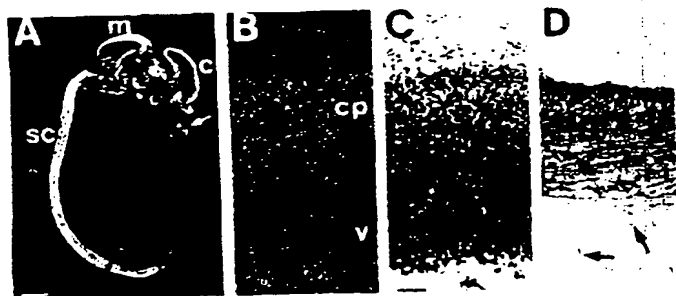
#### TOAD-64 mRNA is enriched in the nervous system and is developmentally regulated

Two different partial sequences from the TOAD-64 gene, representing bases 530–1215 and 1220–1533, were used to study the regulation and distribution of TOAD-64 mRNA. Northern analysis shows that a single band at 4.5 kb representing the TOAD-64 mRNA is developmentally regulated (Fig. 3a). TOAD-64 mRNA is detected in brain at E17. The levels increase up to postnatal day 5 (P5), and subsequently decline to an almost undetectable level in the adult. No TOAD-64 message is detectable in the non-neural tissues sampled (Fig. 3b). The pattern of regulation of the TOAD-64 mRNA parallels that previously described for the TOAD-64 protein (Minturn et al., 1995).

*In situ* hybridization confirms both the developmental regulation and the neural specificity of TOAD-64 mRNA (Fig. 4). TOAD-64 mRNA is abundant throughout the neuraxis at E14. Hybridization signals are high in both the CNS and PNS. In the PNS, sensory and autonomic ganglia express TOAD-64. No hybridization is detected outside of the nervous system. In the CNS, all regions of the brain and spinal cord express TOAD-64, although the distribution within each area indicates that not all cells within the CNS are TOAD-64 positive (see below). As development proceeds, hybridization remains high through the first postnatal week, but then declines to an almost undetectable level in most areas of the brain by postnatal day 14 (P14). The only area of the CNS in which TOAD-64 mRNA is detected in adults is the hippocampus. Antibody to TOAD-64 shows that the adult dentate gyrus contains a small population positive cells, whose position and morphology matches that of the dentate neurons that are born in adult rats (Bayer, 1982). Antibody staining also reveals TOAD-64 protein expression in the primary afferents to the olfactory bulb in adult animals, axons from neurons that continue to be generated in the adult (Graziadei and Monti-



**Figure 4.** *In situ* hybridization confirms the neural specificity of TOAD-64 and demonstrates the broad distribution of TOAD-64 in the developing brain. Parasagittal sections from E14 (A), P0 (B), P5 (C), P14 (D), and P21 (E) rats were hybridized to a  $^{35}$ S-labeled cRNA probe for rat TOAD-64 and the middle subunit of neurofilament (F) as a control. A, At E14, TOAD-64 mRNA is detected throughout the developing brain and spinal cord (sc), and in the dorsal root ganglia (arrow) of the peripheral nervous system. B, At P0 TOAD-64 hybridization is detected throughout the brain, with a particularly high level of signal in the neocortex (c). The hybridization signal in the neocortex is not uniform; labeling is high in the developing cortical plate and far lower in the ventricular zone (arrow) (see also Fig. 6). C, At P5, hybridization is still high in the neocortex as is labeling in the olfactory bulb (o) and pyriform cortex (p). D, At P14, hybridization throughout the brain is reduced. E, By P21, the expression of TOAD-64 mRNA is almost undetectable. F, At P21, neurofilament mRNA is abundantly expressed in differentiated neurons throughout the CNS. Scale bar, 1 mm.



**Figure 5.** In the developing cortex, TOAD-64 is expressed in the cortical plate, which contains postmitotic neurons, but not in the mitotic cells of the ventricular zone. Parasagittal sections of E17 rat embryos were hybridized with TOAD-64 antisense cRNA probe (A and B), and stained with cresyl violet (C), or, sections were stained with TOAD-64 antiserum (D). A, A view of the whole embryo demonstrates the restriction of TOAD-64 mRNA expression to the nervous system. Hybridization is abundant in the spinal cord (sc), midbrain (m), and neocortex (c). Signal is also detected in the olfactory epithelium (arrow). B, An emulsion-dipped section viewed under dark-field illumination shows that in the E17 cortex, hybridization signal is most abundant in the cortical plate (cp). The ventricular zone (v), though very cell dense (compare with panel C), has a far lower density of silver grains. C, Cresyl violet staining shows the relative cell densities of each layer of the E17 neocortex. D, Antibody staining of the cortex shows that the TOAD-64 protein has a very similar distribution to that of the mRNA. Antibody staining is largely restricted to the intermediate zone and the developing cortical plate. The ventricular zone is largely unstained, with the exception of a few process-bearing cells (arrows) that appear to be migrating into the intermediate zone (i).

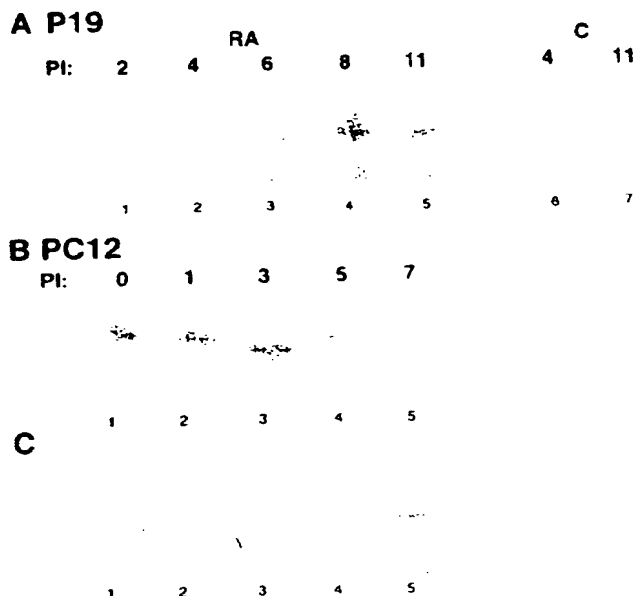
Graziadei, 1978). While we have not examined the nasal sinuses in adult animals, in embryos TOAD-64 mRNA is present in the olfactory epithelium (Fig. 5a).

In a previous report we showed that the TOAD-64 protein is first expressed by neurons shortly after they have completed their final mitosis (Mintum et al., 1995). It was important to determine if this pattern of protein expression reflected transcriptional or translational regulation. *In situ* hybridization shows clearly that TOAD-64 mRNA, like TOAD-64 protein, is not detected in the ventricular zone of the developing brain. For example, in the cortex at E17, the highest level of hybridization is seen in the cortical plate (Fig. 5b), with far lower levels of hybridization in the ventricular and subventricular zones, similar to the distribution of the protein (Fig. 5d). These results indicate that the restricted expression of the protein to newly born neurons is likely to be controlled at the level of gene transcription.

#### TOAD-64 is expressed by P19 and PC12 cells coincident with neuronal differentiation

In the developing brain TOAD-64 is expressed only by cells that have undergone a commitment to a neuronal phenotype. To address the possible association of TOAD-64 expression with neuronal differentiation, we examined the expression of TOAD-64 in two cell lines that can be induced to assume a neuronal phenotype, the embryonal carcinoma cell line, P19, and the rat pheochromocytoma cell line, PC12.

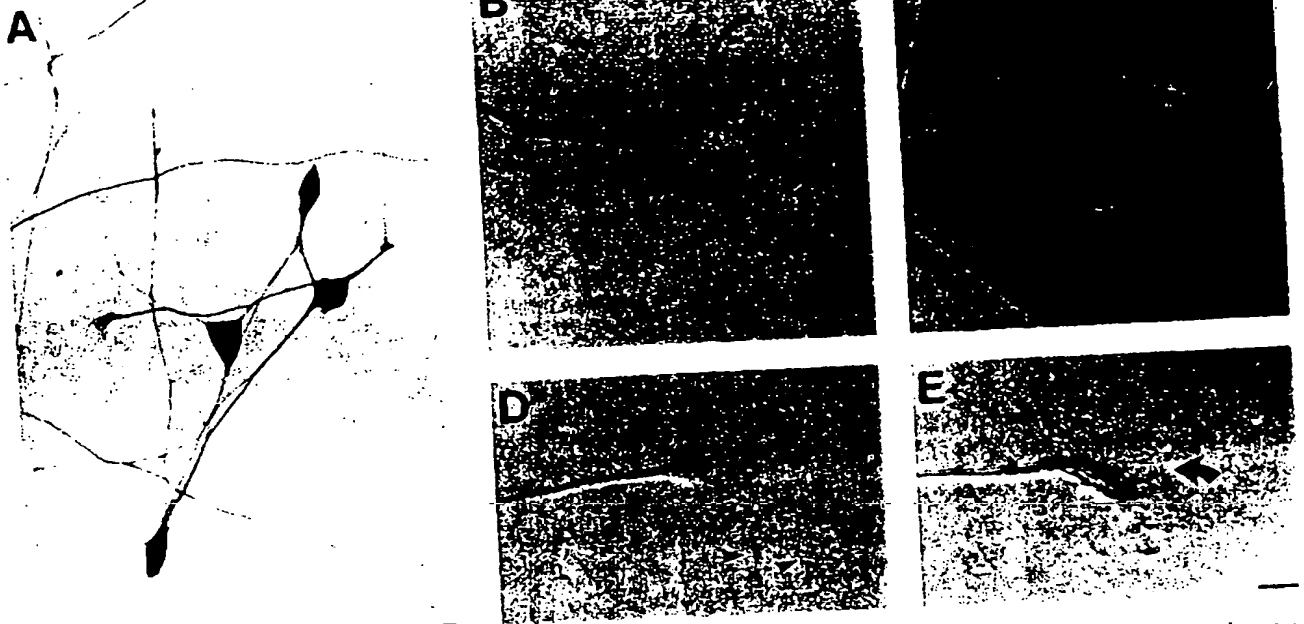
P19 cells are developmentally pluripotent (Edwards and McBurney, 1983). P19 cells exposed to retinoic acid (at greater than  $3 \times 10^{-7}$  M) develop neural properties: by 6 d after exposure to retinoic acid approximately 85% of cells express neuronal markers (McBurney et al., 1988; Staines et al., 1994). We examined undifferentiated and retinoic acid differentiated P19 cells by Northern analysis (Fig. 6a). Prior to neural induction, TOAD-



**Figure 6.** TOAD-64 is regulated in parallel with neuronal differentiation of P19 and PC12 cells. A, Total RNA (40  $\mu$ g/lane) from retinoic acid induced (RA) or control (C) cultures of P19 embryonal carcinoma cells were probed for TOAD-64 mRNA expression. TOAD-64 mRNA is not detected at 2 d after induction (lane 1), but is detected at increasing levels after 4, 6, 8, and 11 d of retinoic acid treatment (lanes 2–5). Control cells at 4 or 11 d in culture do not express TOAD-64 mRNA (lanes 6 and 7). B, Total RNA (35  $\mu$ g/lane) from PC12 pheochromocytoma cells probed for TOAD-64 mRNA shows that prior to (lane 1) or in the days following induction with NGF (PI; lanes 2–5) TOAD-64 mRNA is expressed. C, In contrast to the mRNA, Western blots of protein from uninduced (lane 1) or NGF-induced (lanes 2–5) PC12 cells show that prior to (lane 1) or 1 d after (lane 2) NGF induction, little TOAD-64 protein is detected. Three days following NGF-induction (lane 3) TOAD-64 protein is detected on Western blots and increases gradually thereafter (lanes 4 and 5).

64 mRNA is not expressed, but exposure to retinoic acid induces the expression of TOAD-64 mRNA.

PC12 cells are an adrenal chromaffin-derived tumor cell line (Tischler, 1975; Greene and Tischler, 1976), that, when grown in the absence of nerve growth factor (NGF), proliferate and do not assume neuronal properties. When NGF is added to the culture medium, PC12 cells differentiate into cells with neuronal properties, including the cessation of mitotic activity, neurite extension, and the expression of genes associated with mature neurons (Tischler, 1975; Stein et al., 1988; Vetter and Betz, 1989; Sano et al., 1990). TOAD-64 expression was assayed in uninduced and NGF-induced PC12 cells. In contrast to P19 cells, PC12 cells, in the absence of any induction by NGF, express TOAD-64 mRNA (Fig. 6b). Furthermore, there is no apparent upregulation of mRNA following NGF induction. The disparity between the behavior of TOAD-64 mRNA in these two cell types prompted us to examine the expression of TOAD-64 protein in PC12 cells (Fig. 6c). In the absence of NGF, little TOAD-64 protein is detected. After NGF addition, the amount of TOAD-64 increases at 24 hr and continues to increase up to 5 d. These results suggest that the TOAD-64 protein is involved in the neuronal differentiation of PC12 cells, perhaps in the elaboration of neurites. However, in PC12 cells, in marked contrast to both P19 cells and to neurons *in vivo*, TOAD-64 protein expression is not transcriptionally regulated.



**Figure 7.** TOAD-64 is present in the lamellipodia of growth cones in primary neuronal cultures. *A*, In primary cultures of rat dorsal root ganglion neurons, TOAD-64 is present in the cell bodies and processes of neurons. *B* and *C*, TOAD-64 immunoreactivity is detected throughout the growth cone. The darkest staining for TOAD-64 is seen in the distal part of the neurite, just as the neurite gives rise to the growth cone (asterisks). TOAD-64 immunoreactivity extends into the growth cone itself, with staining present at the leading edge of the lamellipodia (curved arrow in *C*). Even many of the fine filopodia that arise from the growing tip of the neurite contain TOAD-64 protein (arrowheads). *D* and *E*, In contrast to the distribution of TOAD-64 in the growth cone, immunoreactivity for class III  $\beta$ -tubulin is restricted to the axon and only the most proximal portion of growth cones (asterisks). Class III  $\beta$ -tubulin is not observed either in the distal part of the growth cone (curved arrow in *E*) or in the filopodia (arrowheads in *D*). Scale in *A* = 20  $\mu$ m; scale in *B*–*E* = 5  $\mu$ m.

#### TOAD-64 is present in growth cones

To determine whether TOAD-64 might have a role in the mechanisms by which axons advance toward their targets, we examined the distribution of the TOAD-64 protein in neurons in primary culture. In cultures of dorsal root ganglion cells or cortical neurons grown on a polyornithine-laminin substrate, growth cones can be easily identified after 20 hr in culture. Immunohistochemical analysis shows that TOAD-64 is expressed in neuron cell bodies and processes (Fig. 7*a*). TOAD-64 immunoreactivity is present along the full length of the axon into the growth cone. In the growth cone immunoreactivity is most intense at the center of the growth cone, but it also extends through the lamellipodia to the edges of the lamellipodial veil (Figs. 7*b,c*; 8*a,c*). Often a region of particularly intense immunoreactivity for TOAD-64 is observed in the fine filopodial extensions from the growth cone. The distribution of TOAD-64 in the growth cone is different from the cytoskeletal protein, class III  $\beta$ -tubulin (Figs. 7*d,e*; 8*b,d*), which does not extend into the lamellipodia or filopodia.

#### TOAD-64 is reexpressed following peripheral axotomy

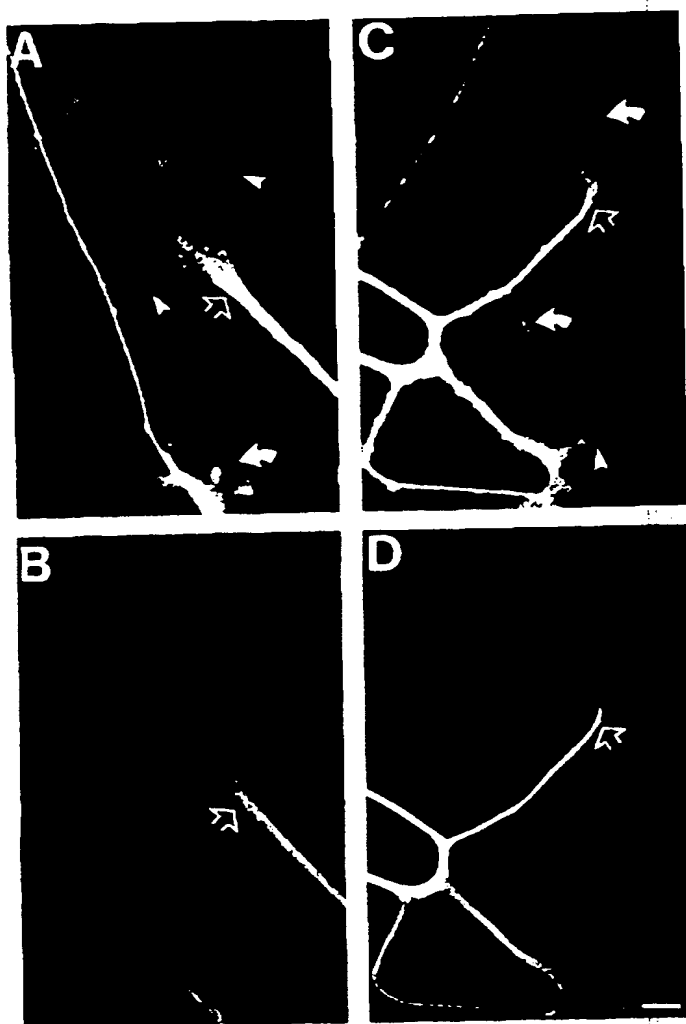
The possibility that TOAD-64 might play a role in the growth of axons led us to examine its expression during axon regeneration in adult animals. Following lesions of the sciatic nerve, sciatic motor neurons with cell bodies in the spinal cord can regenerate axons toward their target muscles. Expression of the TOAD-64 protein by the sciatic motor neurons was studied following a sciatic nerve crush. In normal adult animals, there is no detectable TOAD-64 expression by motor neurons. Follow-

ing sciatic nerve crush, TOAD-64 protein is expressed in sciatic motor neurons (Fig. 9*b*). The unlesioned, contralateral, motor neurons do not express the protein (Fig. 9*a*). A time course of the reexpression of TOAD-64 following nerve injury reveals expression of TOAD-64 protein as early as 1 d after nerve crush.

#### TOAD-64 is a membrane associated protein

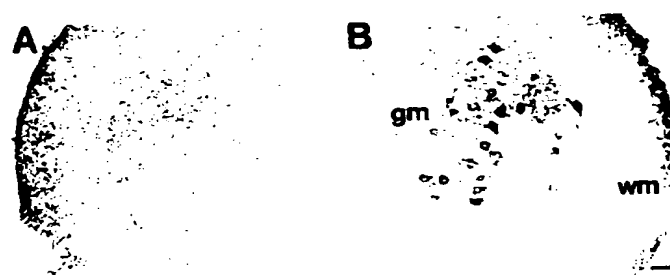
While the sequence of TOAD-64 does not contain a transmembrane domain, biochemical experiments demonstrate that TOAD-64 has a membrane associated form. Two-dimensional gel analyses showed that TOAD-64 partitions with a soluble fraction, which contains cytoplasmic constituents, as well as with a particulate fraction, which contains membrane and cytoskeletal elements. Western blot analysis of a P12 cortical homogenate using antisera to TOAD-64 provides further evidence that TOAD-64 has both soluble and particulate forms (Fig. 10*a*).

To determine the mechanism of association with the pellet fraction, further extractions were performed (Fig. 10). Extraction of the pellet with PBS or with 2 M NaCl releases only 20% of the TOAD-64 from the particulate fraction. TOAD-64 is not bound to the insoluble fraction through  $\text{Ca}^{2+}$ -dependent mechanisms, because extraction of the particulate fraction with EDTA also releases only 20% of the protein. In contrast, treatment of the particulate fraction with 2 M urea releases 50% of the protein, and 90% of the protein is extracted with 1% Triton X-100. Efficient extraction with Triton makes it unlikely that TOAD-64 is associated with the particulate fraction by binding to the Triton insoluble cytoskeleton. The fact that over 90% of the TOAD-64 protein can also be extracted with  $\text{Na}_2\text{CO}_3$  indicates that it is



**Figure 8.** Double labeling shows that TOAD-64 is present in lamellipodia and filopodia of growth cones. Primary cultures of dorsal root ganglion neurons were stained for TOAD-64, visualized with FITC (A and C), and for class III  $\beta$ -tubulin, visualized with Texas red (B and D). The same field is shown under FITC optics for TOAD-64 in A and under Texas red optics for class III  $\beta$ -tubulin in B. Similarly, the same field is shown in C and D. A and C, TOAD-64 immunoreactivity is detected throughout the neurite and into the growth cone. Intense staining for TOAD-64 is seen in the distal part of the neurite, just as the neurite gives rise to the growth cone (open arrows). TOAD-64 immunoreactivity extends into the growth cone itself, with staining often present at the leading edge of the lamellipodia (curved arrows). Many of the fine filopodia that arise from the growing tip of the neurite contain TOAD-64 protein (arrowheads). B and D, In contrast to the distribution of TOAD-64 in the growth cone, immunoreactivity for class III  $\beta$ -tubulin is restricted to the neurite and only the most proximal portion of growth cones (open arrows). Class III  $\beta$ -tubulin is not observed either in the distal part of the growth cone or in the filopodia. Scale = 10  $\mu$ m.

either a peripheral membrane protein or trapped within vesicles. Both the amino acid sequence deduced from the nucleic acid sequence, which does not contain a putative transmembrane domain, and the immunohistochemical localization of the protein to the cytoplasm (Minturn et al., 1995), further indicate that TOAD-64 is an intracellular protein. Together these data show that the particulate form of TOAD-64 is likely to be tightly

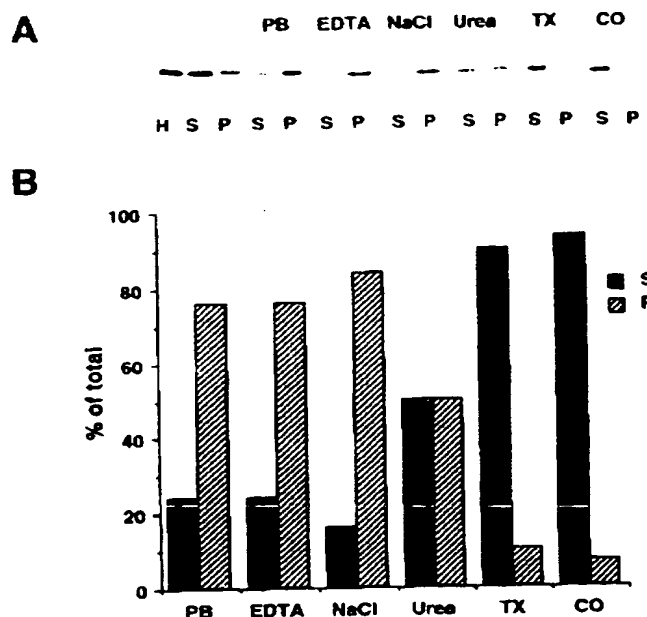


**Figure 9.** In adult animals, TOAD-64 is reexpressed in spinal cord motor neurons following a peripheral axotomy. Five days following sciatic nerve crush, TOAD-64 is detected ipsilateral to the lesion (B), but not contralateral to the lesion (A). A, If the sciatic nerve is intact, antisera to TOAD-64 does not stain motor neurons in the ventral horn of the spinal cord. B, The sciatic motor neurons on the side of the nerve crush show staining with TOAD antisera, indicating a reexpression of the protein after injury, coincident with axon regeneration. wm = white matter; gm = gray matter; Scale bar, 100  $\mu$ m.

associated with a membrane protein, and not itself a transmembrane protein.

## Discussion

We report here the full length sequence and expression pattern of a gene, TOAD-64, that is initially transcribed at the earliest stages in neuronal differentiation. Neither the TOAD-64 protein nor its mRNA is expressed by mitotically active progenitors in the ventricular zone, but both are expressed by postmitotic neu-



**Figure 10.** Extraction conditions for TOAD-64 demonstrate that it is membrane associated. A, Homogenates from P12 rat cerebral cortex (H) were separated into soluble (S) and pellet (P) fractions by centrifugation at 100,000  $\times$  g for 1 hr. The pellet fraction was rehomogenized in PBS and divided into six separate aliquots that were reprecipitated. After resuspending the aliquots in either 0.1 M  $\text{Na}_2\text{CO}_3$ , pH 11 (CO), PBS (PB), PBS+2 M NaCl (NaCl), PBS+10 mM EDTA, PBS+2 M urea, or PBS+1% Triton X-100, soluble and pellet fractions were once again generated. Immunoblots of the protein extracts were then probed with the antibody to TOAD-64. B, The relative amount of TOAD-64 in the soluble or pellet fractions was quantified using densitometry.

rons. TOAD-64 is one of the few known genes that is turned on coincident with the very earliest events in neuronal differentiation. The regulation of TOAD-64 in parallel with axon elongation in several different paradigms, together with its localization to lamellipodia and filopodia of growth cones, suggests a role for this protein in axonogenesis.

The TOAD-64 gene encodes a deduced protein with a molecular mass of 62,364 and pI of 6.34, almost identical to the estimates for the protein from two-dimensional gels (Geschwind and Hockfield, 1989). The TOAD-64 gene shows significant homology to the *C. elegans unc-33* gene. *unc-33* was originally isolated in a screen for motorically uncoordinated mutants. Further histological analysis revealed that all neurons in *unc-33* mutants have defective patterns of axon outgrowth (Hedgecock et al., 1985; Li et al., 1992). In *unc-33* mutants, axons terminate prematurely, have abnormal branch points and follow inappropriate routes (Hedgecock et al., 1985; Desai et al., 1988; Siddiqui and Culotti, 1991; Li et al., 1992). Based on axonal morphology, it was predicted that *unc-33* would encode a cytoskeletal protein, such as a microtubule associated protein (Hedgecock et al., 1985), but subsequent sequence analysis revealed that, like TOAD-64, *unc-33* showed no homology to any protein already in the database (Li et al., 1992). The *unc-33* product is predicted to have several potential phosphorylation sites, consistent with the fact that the TOAD-64 gene sequence contains consensus sites for serine, threonine, and tyrosine phosphorylation. In addition, *unc-33* lacks a transmembrane region or signal peptide, as does TOAD-64. The major difference between *unc-33* and TOAD-64 is that *unc-33* has three transcripts, while we have detected only one for TOAD-64. The size of the largest predicted *unc-33* protein, 90 kDa, is significantly larger than TOAD-64; however, the *unc-33* protein(s) itself has not been isolated or characterized. Nevertheless, the sequence and predicted structural similarities between the *unc-33* gene product and TOAD-64 suggest that they are related, and perhaps homologous, proteins from evolutionarily distant species. This provides support for the possibility that TOAD-64 may, like *unc-33*, play a critical role in axon outgrowth in rodents.

Our previous work led to the suggestion that TOAD-64 might participate in early neuronal differentiation, such as in cell migration or axon outgrowth (Minturn et al., 1995). Antibodies to TOAD-64 showed that the protein is first expressed by neurons shortly after the final mitosis, at a time when neurons are just beginning their migration out of the ventricular zone. TOAD-64 expression precedes that of several neuronal markers, including neurofilament and MAP2. Double labeling with an antibody to class III  $\beta$ -tubulin showed that all postmitotic neurons express TOAD-64. The *in situ* hybridization analyses presented here indicate that the TOAD-64 gene, like the protein, is not expressed in the region occupied by progenitor cells, but is first detected outside of the ventricular zone. These data on the expression of TOAD-64 mRNA demonstrate that the regulation of TOAD-64 protein in the developing brain is at the level of gene transcription. Unlike most previously described neuronal genes and proteins, TOAD-64 mRNA is downregulated to an almost undetectable level in most regions of the adult brain. The decline in TOAD-64 expression during the second postnatal week coincides with the end of the major period of axon growth, suggestive of a role in neurite extension.

TOAD-64 expression in cell lines further underscores its regulation in parallel with neuronal differentiation and axon outgrowth. TOAD-64 mRNA is not detected in the embryonal car-

cino cell line, P19, prior to the induction of a neuronal phenotype. Induction of neuronal properties by retinoic acid induces the expression of TOAD-64 mRNA. In PC12 cells, neuronal differentiation apparently increases the rate of translation or the stability of the TOAD-64 mRNA or protein without altering the constitutive expression of TOAD-64 mRNA. This kind of protein regulation is similar to that observed for other neuronal proteins in PC12 cells (Sharma et al., 1993). Although the mechanism of regulation of protein levels is different in P19 and PC12 cells, it appears likely in both cases that TOAD-64 protein plays a role in the acquisition of a neuronal phenotype, potentially in mediating neurite outgrowth.

A role in neurite outgrowth is also suggested by the localization of TOAD-64 to the lamellipodia and filopodia at the advancing edge of growth cones. Moreover, further evidence supporting such a role comes from our experiments on axon regeneration. Reexpression during axon regeneration would be predicted for a protein required for axon outgrowth or navigation. During development, TOAD-64 is expressed at a high level in the spinal cord. In the normal adult spinal ventral horn, as in most other areas of the adult CNS, there is little detectable TOAD-64. Motor neurons with axons in the sciatic nerve reexpress TOAD-64 as they regenerate to find targets following a sciatic nerve lesion. Protein upregulation during axon regeneration has also been demonstrated for GAP-43 (Skene and Willard, 1981), a protein known to be associated with axonal elongation. Also consistent with a role in axonogenesis is the expression of TOAD-64 in areas of the adult brain that have the capacity for ongoing neurogenesis and axon growth.

A membrane-associated protein expressed during axonal extension and localized at lamellipodia would be optimally situated to participate in the signal transduction processes that permit growing axons to choose correct routes and targets. The nucleic acid sequence of TOAD-64 encodes a protein that lacks a signal sequence or a transmembrane domain, dictating that the TOAD-64 protein is cytoplasmic. However, extraction experiments demonstrate that the protein has both soluble and membrane-associated forms. The mechanism by which TOAD-64 associates with the membrane fraction is not apparent from the sequence alone. Biochemical experiments show that conditions that extract transmembrane proteins and proteins associated with them can release TOAD-64 from a crude membrane preparation. It is therefore likely that TOAD-64 binds noncovalently with high affinity to a membrane protein. A membrane localization might be predicted for a protein that participated in the cellular machinery by which axons navigate to reach their targets. While definitive demonstration of the function of TOAD-64 still lies before us, the data presented here and previously (Geschwind and Hockfield, 1989; Minturn et al., 1995) suggest that TOAD-64 could be a central element in the complex machinery underlying axonal outgrowth and pathfinding.

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# Identification and Characterization of a Retinoic Acid-regulated Human Homologue of the *unc-33*-like Phosphoprotein Gene (hUlip) from Neuroblastoma Cells\*

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A cDNA, 7G1, was isolated from retinoic acid (RA) differentiated neuroblastoma cells whose expression was high in human fetal brain and spinal cord mRNA but undetectable in adult brain or non-neuronal tissues. Sequence analysis indicates that 7G1 is homologous to the *Caenorhabditis elegans* gene *unc-33*. A 5.5-kilobase pair full-length cDNA from a human fetal brain cDNA library contains an 1710-base pair open reading frame. Because the predicted 570 amino acid sequence of 7G1 shares 98% identity with the murine Ulip gene product, an *unc-33*-like-phosphoprotein, we refer to 7G1 as the human Ulip (hUlip). hUlip is also similar to the bacterial enzyme D-hydantoinase and the recently described vertebrate gene products CRMP62, TOAD-64, CRMP1, CRMP2, and mUNC. RA stimulates an increase in hUlip mRNA that is transcriptionally regulated. RA stimulates an increase in polypeptides of 58, 60, 65, and 70 kDa with the 58- and 65-kDa species being dephosphorylated forms of the 60- and 70-kDa species. This study presents a model in which to study the regulation and expression of the hUlip gene, a member of an emerging family of molecules that potentially mediates signals involved in axonal outgrowth.

Human neuroblastomas (NB)<sup>1</sup> constitute a unique *in vitro* model in which to explore the cellular and molecular mechanisms that regulate the growth and differentiation of human peripheral nervous system tissue (1, 2). In the presence of biological response modifiers such as retinoic acid (RA) (3), increases in cAMP (4), phorbol esters (5), and interferons (6), the proliferation of these tumor cells is decreased, and there is an increase in neurite extension and neurotransmitter expression, and cells acquire some electrophysiologic properties similar to normal neurons. Several NB cell lines treated *in vitro* with RA increase Trk gene expression are induced to change the expression of several molecular markers, thus recapitulating steps of the normal embryonic development (7-10). Our studies on the mechanisms of RA-induced differentiation of NB cells show that RA induces an increase in TrkB mRNA transcription as well as protein production. Furthermore, in neuroblastoma cell lines constitutively producing BDNF, RA-in-

duced TrkB expression leads to an activation of the TrkB signal transduction pathway that stimulates neurite extension and differentiation (11). These data suggest that RA may stimulate gene expression and lead to the activation of signal transduction pathways usually suppressed in the transformed NB cell.

To isolate molecular effectors important during activation of the *in vitro* differentiation of human NB cells, we screened a cDNA library made from NB cells treated for 14 days with RA and identified several genes whose expression changed during RA treatment. One such gene named 7G1 was isolated and found to detect a 5.5-kb mRNA species that was markedly increased after RA treatment of NB cells (12, 13). DNA sequence analysis indicates that 7G1 shares a striking homology with the mouse *unc-33*-like-phosphoprotein, Ulip/*unc-33*-like-phosphoprotein gene (14) and the recently described rat CRMP-4 (collapsin response-mediated protein) (15). We therefore renamed our gene hUlip as a human homologue of the *Caenorhabditis elegans* uncoordinated 33 (*unc-33*) gene. hUlip also has homology with the 3' region of the *C. elegans* gene *unc-33* (16) and the bacterial enzyme D-hydantoinase as well as a series of highly conserved genes, among several different vertebrate species including the avian CRMP62 (17), and mUNC,<sup>2</sup> the rat TOAD-64 (18, 19), and two other human sequences named hCRMP1 and hCRMP2 (17). This paper describes the isolation of a full-length hUlip cDNA from a human fetal brain cDNA library, hUlip expression in human tissues during development, and regulation of the expression of the hUlip gene and protein in during RA-induced differentiation of NB tumor cells.

## MATERIALS AND METHODS

**Cell Culture**—The NB cell lines SMS-KCNR (KCNR) (20), SH-SY5Y (SY5Y) (21), and NGP (22) were cultured as described previously (11, 23, 24). Cells were treated with the indicated concentration of all-trans-retinoic acid (Sigma) or control solvent for the indicated times.

**Isolation of hUlip cDNA**—A 3.7-kb fragment of hUlip (7G1) was cloned from RA-treated KCNR cDNA library by differential hybridization method as described previously (12, 13). To isolate the full-length hUlip cDNA, five hundred thousand phage from a human fetal brain  $\lambda$  ZAPII cDNA library (Stratagene, La Jolla, CA) were screened using a <sup>32</sup>P-labeled 800-bp fragment of 7G1. Plaque purified phage clones were converted from  $\lambda$  ZAPII vector to pBluescript SK(-) plasmid according to the manufacturer's recommendation, and two overlapping clones, clone 1 and clone 2, contained the entire hUlip coding region. cDNA sequences were determined by dideoxynucleotide chain termination sequencing reactions (25) using synthetic oligonucleotides. Each clone was sequenced in both directions and sequencing confirmed that clones 1 and 2 are completely identical with the original 800-bp 7G1 cDNA fragment.

**RNA Analysis and *In Vitro* Transcription Analysis**—RNA isolations and hybridizations were performed as described previously (12, 13). Typically 25  $\mu$ g of total RNA was analyzed by Northern blot analysis

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) Y07818.

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The abbreviations used are: NB, neuroblastoma; RA, retinoic acid; kb, kilobases; bp, base pairs; PAGE, polyacrylamide gel electrophoresis; BDNF, brain-derived neurotrophic factor.

<sup>2</sup> S. Tontsch, unpublished observation.

and a  $^{32}$ P-labeled 5-kb insert of hUlip clone 1 or GAPDH. Washing conditions were as described (24). Membranes were exposed to X-Omat AR film at  $-70^{\circ}\text{C}$  using an intensifying screen. Nuclear RUN-ON assay was performed as described previously (26).

**In Vitro Transcription and Translation of the hUlip Gene**—A pBlue-script SK(-) phagemid containing hUlip cDNA clone 2 served as a template for *in vitro* transcription using the T7 polymerase Ribo MAX kit (Promega), and the resulting mRNA was translated *in vitro* with rabbit reticulocyte lysate (Promega) in the presence of [ $^{35}$ S]methionine. For the immunoprecipitation, the translated product was diluted 10-fold with protein extraction buffer (1% Nonidet P-40, 20 mM Tris, pH 8.0, 137 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 0.15 unit/ml of aprotinin, 20 mM leupeptin, 1 mM sodium vanadate, 10 mM NaF) and incubated with anti-peptide A serum and protein A-Sepharose CL-4B (Pharmacia Biotech Inc.) overnight at  $4^{\circ}\text{C}$ . After washing with extraction buffer, immunoprecipitates were eluted by boiling 5 min in 1  $\times$  protein sample buffer (1  $\times$  = 62.5 mM Tris, pH 6, 10% glycerol, 2.3% SDS, 5% 2-mercaptoethanol, and 0.25% bromophenol blue) and analyzed by SDS-PAGE.

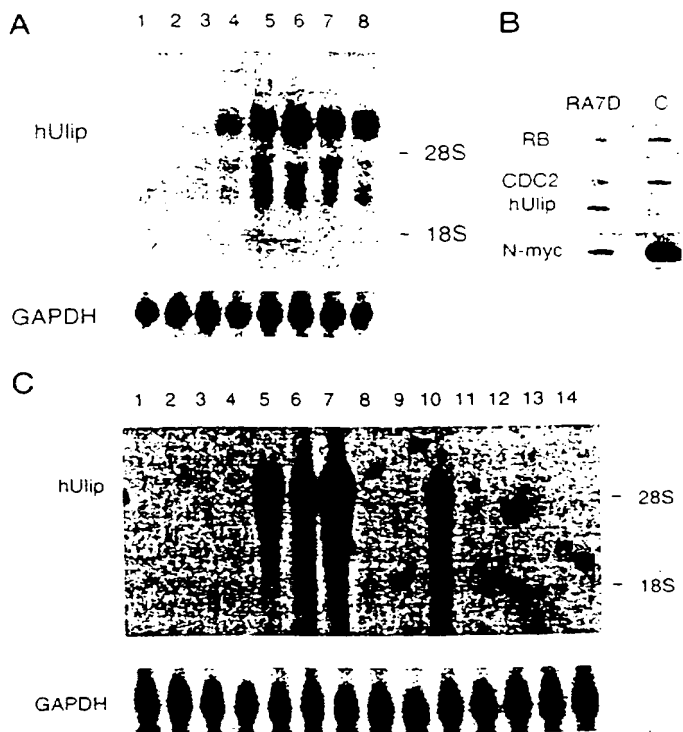
**Protein Analysis**—Cells ( $1 \times 10^6$ ) were plated in 100-cm $^2$  dishes for 24 h and treated with indicated concentrations of RA or control solvent for the indicated times. Cells ( $1 \times 10^7$ ) were lysed in 1 ml of protein extraction buffer at  $4^{\circ}\text{C}$  for 30 min, insoluble material was removed by centrifugation at 10,000  $\times$  g, and protein concentration was determined by protein assay kit (Bio-Rad). 20  $\mu\text{g}$  of proteins were electrophoresed on 10% SDS-polyacrylamide gels (PAGE) and transferred to nitrocellulose. Filters were blocked with 5% nonfat dry milk in Tris-buffered saline with 0.5% Tween 20 (TBST) and hULIP proteins were detected using an antiserum (1:1000) raised against Peptide A. Peptide A corresponds to amino acids 499–511 of hULIP and ULIP. The anti-peptide A serum is specifically blocked by Peptide A and not the corresponding peptide in the TOAD-64/CRMP2 gene.<sup>3</sup> The anti-peptide A serum was originally described to detect the TOAD-64 protein, which is now known to be Ulip/CRMP4 (14, 15). The blots were washed, and bound antibodies were detected with the ECL kit (Amersham Corp.).

For protein phosphatase treatment, after cells were lysed with lysis buffer (1% Nonidet P-40, 50 mM Tris, pH 8.5, 137 mM NaCl, 2 mM MgCl $_2$ , 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.15 unit/ml of aprotinin, 20 mM leupeptin, 0.1 mM dithiothreitol), 20  $\mu\text{g}$  of protein extracts were incubated with 15 units of calf intestinal alkaline phosphatase (Boehringer Mannheim) at  $37^{\circ}\text{C}$  for 30 min and analyzed by immunoblot as described above.

## RESULTS

**Expression and Regulation of hUlip**—The hUlip gene was originally isolated as a partial cDNA clone (7G1) from a cDNA library generated from NB cells that had been treated for 14 days with RA (12). To study the kinetics of RA inducibility of the hUlip gene, we analyzed RNA isolated from SMS-KCNR NB cells at various times after treatment. An increase in hUlip steady-state mRNA levels was not detected after 15 min of RA treatment (Fig. 1A, lane 2). However, within 1 h a small increase in hUlip expression was detected (Fig. 1A, lane 3) that was markedly increased after 2 days of RA treatment (Fig. 1A, lane 4). hUlip expression peaked between 4–8 days (Fig. 1A, lanes 5 and 6) and remained relatively unchanged up to 18 days after RA treatment (Fig. 1A, lanes 7 and 8). At this time, the transcriptional induction of hUlip gene was approximately 20-fold higher in RA-treated cells than in control cells (Fig. 1B). This is not due to a generalized increase in transcription stimulated by RA because the specific transcription of the retinoblastoma and CDC2 genes was decreased less than 2-fold after RA treatment, whereas N-myc transcription was reduced approximately 50-fold by RA as has been previously described (24). The transcriptional regulation of hUlip is consistent with a previous observation that RA stimulated a 2-fold increase in 7G1 transcription in nuclei from cells treated for 2 days with RA and a 22-fold induction in nuclei from cells after 14 days of RA treatment (13).

A Northern analysis of normal human fetal and adult tissues



**FIG. 1. Expression and regulation of hUlip mRNA.** A, Northern analysis of hUlip expression in the NB cell line SMS-KCNR evaluated during a time course of treatment with RA. Lane 1, control cells; lane 2, 15 min; lane 3, 2 h; lanes 4–8, 2, 4, 8, 10, and 18 days of RA treatment, respectively. B, nuclear RUN-ON assay performed on isolated nuclei from KCNR after 7 days of culture in standard condition or in presence of RA 5  $\mu\text{M}$ . C, Northern analysis of hUlip expression in normal fetal and adult tissues. Lane 1, adult skeletal muscle; lane 2, fetal limb tissue; lane 3, adult muscle; lane 4, neuroblastoma cell line SMS-KCNR; lane 5, RA-treated SMS-KCNR cells; lane 6, 59-day-old fetal human brain; lane 7, 135 days old fetal human brain; lanes 8 and 9, adult brain (unknown areas); lanes 10 and 11, fetal human spinal cord and adult dorsal root ganglion tissues; lane 12, adult peripheral nerve tissue; lanes 13 and 14, adrenal and pancreatic tissues. RB, retinoblastoma.

indicated that hUlip mRNA is detected in 59-day fetal brain (Fig. 1C, lane 6) and an increased level of hUlip mRNA is observed in a 135-day brain sample (lane 7). However, two different samples of adult brain mRNA (regions undefined) did not express hUlip mRNA (lanes 8 and 9). Detectable levels of hUlip mRNA are also found in fetal spinal cord mRNA but not in adult dorsal root ganglion or peripheral nerve mRNA (Fig. 1C, lanes 10, 11, and 12). Expression of hUlip was not detected in RNA samples from adult muscle, fetal limb, adrenal, or pancreas (Fig. 1C, lanes 1, 2, 3, 13, and 14, respectively).

**Sequence Analysis and Comparisons**—The 7G1 clone contained a 3.7-kb cDNA, and recent sequence analysis comparisons showed that 7G1 shared homology with the *C. elegans* gene *unc-33*. The highest homology matched a 3' region of the *unc-33* cDNA. Using a polymerase chain reaction generated 800-bp DNA fragment to the most 5' region of the 7G1 cDNA as a probe, we isolated a full-length cDNA insert of 5.5 kb from a human fetal brain library. Sequencing of both strands and computer analysis of the 5.5-kb cDNA indicated an open reading frame of 1710 bp predicted a 62-kDa polypeptide with a pI of 6.4 (Fig. 2). Sequencing indicated that 7G1 was identical to this 5.5-kb clone in the 3' region of the molecule. A more detailed nucleotide data base search revealed several highly homologous sequences. The highest homology, 98% identity at

<sup>3</sup> S. Hockfield, personal communication.



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GGG CGG AGG CTG CGG CGC GGC CAG CGC ATT CAC TCC ACC TGA TC  
9 18 27 36 45  
GGC GCT GTG CGC TGA GGA AGG CGC GGG CGA GCC GGA GCA GAA GAA GGA GGG AGG C  
60 78 87 96 105  
CCA GCC GCT GCA GCC ACC ACC GCC ACC ATG TCC TAC CAA GGC AAG AAG AAC ATC CCG  
117 126 135 144 153 162 171 180 189 198 207 216 225 234 243 252 261 270 279 288 297 306 315 324 333 342 351 360 369 378 387 396 405 414 423 432 441 450 459 468 477 486 495 504 513 522 531 540 549 558 567 576 585 594 603 612 621 630 639 648 657 666 675 684 693 702 711 720 729 738 747 756 765 774 783 792 801 810 819 828 837 846 855 864 873 882 891 900 909 918 927 936 945 954 963 972 981 990 1000 1009 1018 1027 1036 1045 1054 1063 1072 1081 1090 1100 1109 1118 1127 1136 1145 1154 1163 1172 1181 1190 1200 1209 1218 1227 1236 1245 1254 1263 1272 1281 1290 1300 1309 1318 1327 1336 1345 1354 1363 1372 1381 1390 1400 1409 1418 1427 1436 1445 1454 1463 1472 1481 1490 1500 1509 1518 1527 1536 1545 1554 1563 1572 1581 1590 1600 1609 1618 1627 1636 1645 1654 1663 1672 1681 1690 1700 1709 1718 1727 1736 1745 1754 1763 1772 1781 1790 1800 1809 1818 1827 1836 1845 1854 1863 1872 1881 1890 1900 1909 1918 1927 1936 1945 1954 1963 1972 1981 1990 2000 2009 2018 2027 2036 2045 2054 2063 2072 2081 2090 2100 2109 2118 2127 2136 2145 2154 2163 2172 2181 2190 2200 2209 2218 2227 2236 2245 2254 2263 2272 2281 2290 2300 2309 2318 2327 2336 2345 2354 2363 2372 2381 2390 2400 2409 2418 2427 2436 2445 2454 2463 2472 2481 2490 2500 2509 2518 2527 2536 2545 2554 2563 2572 2581 2590 2600 2609 2618 2627 2636 2645 2654 2663 2672 2681 2690 2700 2709 2718 2727 2736 2745 2754 2763 2772 2781 2790 2800 2809 2818 2827 2836 2845 2854 2863 2872 2881 2890 2900 2909 2918 2927 2936 2945 2954 2963 2972 2981 2990 3000 3009 3018 3027 3036 3045 3054 3063 3072 3081 3090 3100 3109 3118 3127 3136 3145 3154 3163 3172 3181 3190 3200 3209 3218 3227 3236 3245 3254 3263 3272 3281 3290 3300 3309 3318 3327 3336 3345 3354 3363 3372 3381 3390 3400 3409 3418 3427 3436 3445 3454 3463 3472 3481 3490 3500 3509 3518 3527 3536 3545 3554 3563 3572 3581 3590 3600 3609 3618 3627 3636 3645 3654 3663 3672 3681 3690 3700 3709 3718 3727 3736 3745 3754 3763 3772 3781 3790 3800 3809 3818 3827 3836 3845 3854 3863 3872 3881 3890 3900 3909 3918 3927 3936 3945 3954 3963 3972 3981 3990 4000 4009 4018 4027 4036 4045 4054 4063 4072 4081 4090 4100 4109 4118 4127 4136 4145 4154 4163 4172 4181 4190 4200 4209 4218 4227 4236 4245 4254 4263 4272 4281 4290 4300 4309 4318 4327 4336 4345 4354 4363 4372 4381 4390 4400 4409 4418 4427 4436 4445 4454 4463 4472 4481 4490 4500 4509 4518 4527 4536 4545 4554 4563 4572 4581 4590 4600 4609 4618 4627 4636 4645 4654 4663 4672 4681 4690 4700 4709 4718 4727 4736 4745 4754 4763 4772 4781 4790 4800 4809 4818 4827 4836 4845 4854 4863 4872 4881 4890 4900 4909 4918 4927 4936 4945 4954 4963 4972 4981 4990 5000 5009 5018 5027 5036 5045 5054 5063 5072 5081 5090 5100 5109 5118 5127 5136 5145 5154 5163 5172 5181 5190 5200 5209 5218 5227 5236 5245 5254 5263 5272 5281 5290 5300 5309 5318 5327 5336 5345 5354 5363 5372 5381 5390 5400 5409 5418 5427 5436 5445 5454 5463 5472 5481 5490 5500 5509 5518 5527 5536 5545 5554 5563 5572 5581 5590 5600 5609 5618 5627 5636 5645 5654 5663 5672 5681 5690 5700 5709 5718 5727 5736 5745 5754 5763 5772 5781 5790 5800 5809 5818 5827 5836 5845 5854 5863 5872 5881 5890 5900 5909 5918 5927 5936 5945 5954 5963 5972 5981 5990 6000 6009 6018 6027 6036 6045 6054 6063 6072 6081 6090 6100 6109 6118 6127 6136 6145 6154 6163 6172 6181 6190 6200 6209 6218 6227 6236 6245 6254 6263 6272 6281 6290 6300 6309 6318 6327 6336 6345 6354 6363 6372 6381 6390 6400 6409 6418 6427 6436 6445 6454 6463 6472 6481 6490 6500 6509 6518 6527 6536 6545 6554 6563 6572 6581 6590 6600 6609 6618 6627 6636 6645 6654 6663 6672 6681 6690 6700 6709 6718 6727 6736 6745 6754 6763 6772 6781 6790 6800 6809 6818 6827 6836 6845 6854 6863 6872 6881 6890 6900 6909 6918 6927 6936 6945 6954 6963 6972 6981 6990 7000 7009 7018 7027 7036 7045 7054 7063 7072 7081 7090 7100 7109 7118 7127 7136 7145 7154 7163 7172 7181 7190 7200 7209 7218 7227 7236 7245 7254 7263 7272 7281 7290 7300 7309 7318 7327 7336 7345 7354 7363 7372 7381 7390 7400 7409 7418 7427 7436 7445 7454 7463 747

hUlip	1	MSYQGKKNIP	RITS	DRLLIK	GGRIVNDDQS	FYADIYMEDG	LIKQIGDNVI	50
Ulip		MSYQGKKNIP	RITS	DRLLIK	GGRIVNDDQS	FYADIYMEDG	LIKQIGDNLI	
hUlip	51	VPGGVKTIEA	NGKMVTPGGI	DVHTHFQMPY	KGMITVDEFF	QCTKAALAGG		100
Ulip		VPGGVKTIEA	NGKMVTPGGI	DVHTHFQMPY	KGMITVDEFF	QCTKAALAGG		
hUlip	101	TTMIIDHWVP	EPES	SLTEAY	EKWREWADGK	SCCDYALHVD	IAHWNDSVKQ	150
Ulip		TTMIIDHWVP	EPES	SLTEAY	EKWREWADGK	SCCDYALHVD	ITHWNSVKQ	
hUlip	151	EVQNLKDKG	VNSFMVVMAY	KDLYQVSNTE	LYEIFTCLGE	LGATAQVHAE		200
Ulip		EVQSLSKERG	VNSFMVVMAY	KDLYQVSNTE	LYEIFTCLGE	LGATAQVHAE		
hUlip	201	NGDIIAQEQT	RMLEMGITGP	EGHVLSPREE	LEAEAVFHAI	TIASQTNCP		250
Ulip		NGDIIAQEQA	RMLEMGITGP	EGHVLSPREE	LEAEAVFHAI	TIASQTNCP		
hUlip	251	YVTKVMSKSA	ADLISQARKK	GNVVFGEPI	ASLGIDGTHY	WSKNWAKAAA		300
Ulip		YVTKVMSKSA	ADLISQARKK	GNVVFGEPI	ASLGIDGTHY	WSKNWAKAAA		
hUlip	301	FVTSPPLSE	PTTDPYINSL	LASGDLQLSG	SAHCTFSTAQ	KAIGKDNFTA		350
Ulip		FVTSPPLSE	PTTDPYINSL	LASGDLQLSG	SAHCTFSTAQ	KAIGKDNFTA		
hUlip	351	IPEGTNGVEE	RMSVIWDKAV	ATGKMDENQF	VAVTSTNAAK	IFNLVPRKGR		400
Ulip		IPEGTNGVEE	RMSVIWDKAV	ATGKMDENQF	VAVTSTNAAK	IFNLVPRKGR		
hUlip	401	ISVGSDDSLV	IWDPPDAVKIV	SAKNHQSAB	YNIFEGMELR	GAPLVVICQG		450
Ulip		IAVGSDDSLV	IWDPPDAVKIV	SAKNHQSAB	YNIFEGMELR	GAPLVVICQG		
hUlip	451	KIMLEDGNLH	VTQAGAGFIP	CSPEFSDYVYK	RIKARRQYAD	LHAVERGMVD		500
Ulip		KIMLEDGNLH	VTQAGAGFIP	CSPEFSDYVYK	RIKARRQYAD	LHAVERGMVD		
hUlip	501	GPVFDLTTTP	KGGTPAGSAR	GSPTRENPPV	RNLHQSGFSL	SGPQNTLGR		550
Ulip		GPVFDLTTTP	KGGTPAGSTR	GSPTRENPPV	RNLHQSGFSL	SGPQNTLGR		
hUlip	551	BASKRIVAPP	GGRSNITSLS					570
Ulip		BASKRIVAPP	GGRSNITSLS					

FIG. 3. Sequence comparison between hUlip and Ulip. Comparison of hUlip protein sequence with the highly related mouse Ulip protein showing 98% of identity in amino acid residues. Mismatched amino acid residues were indicated by asterisks. Shaded boxes represent the consensus sites for protein kinases such as protein kinase C (Ser<sup>14</sup>, Ser<sup>147</sup>, Thr<sup>322</sup>, Ser<sup>121</sup>, Thr<sup>509</sup>, Ser<sup>515</sup>, and Ser<sup>553</sup>), casein kinase II (Thr<sup>24</sup>, Ser<sup>115</sup>, Ser<sup>131</sup>, Ser<sup>177</sup>, Thr<sup>218</sup>, Ser<sup>226</sup>, Ser<sup>259</sup>, Thr<sup>312</sup>, Ser<sup>405</sup>, Ser<sup>427</sup>, and Thr<sup>543</sup>), protein kinase A (Ser<sup>363</sup>, Ser<sup>522</sup>, and Ser<sup>553</sup>), and proline-directed kinases (Ser<sup>308</sup>, Thr<sup>313</sup>, and Ser<sup>172</sup>). Potential N-myristoylation sites were indicated by dots.

protein level, was observed with the mouse *unc-33*-like phosphoprotein Ulip (14) (Fig. 3). The human sequences, hCRMP1 and hCRMP2 (17), showed a lower level of homology ranging from 68 (hCRMP2) to 75% (hCRMP1) (data not shown).

Amino acid sequence comparisons of the Ulip gene revealed domains within the Ulip protein that share significant homologies with the D-hydantoinase protein from *Pseudomonas putida* (14). During the process of sequence analysis, we noted that the hUlip gene product shared a significant number of conserved amino acid residues with the protein sequence of the *Bacillus stearothermophilus* D-hydantoinase enzyme (36% homology) and the *C. elegans unc-33* gene product (30% homology). By alignment of these three proteins we identified four conserved internal domains, A, B, C, and a D region of homology within C in which the level of identity was significantly higher than the average (60–76%) (Fig. 4). Further analysis is required to assess if these regions may be significant in the function of the hUlip protein.

**Analysis of hULIP Protein Expression and Regulation**—To identify the hUlip gene product, we prepared *in vitro* translated hULIP protein. The 2-kb hUlip mRNA that encompassed the coding region of hUlip was transcribed by T7 polymerase *in vitro* from the pBluescript II SK(–) hUlip cDNA clone 2 and translated into protein using a rabbit reticulocyte lysate in the presence of [<sup>35</sup>S]methionine and analyzed by SDS-PAGE (Fig. 5A). [<sup>35</sup>S]Methionine-labeled proteins ranging from 35 to 60 kDa are detected, and the 60-kDa labeled protein corresponds

to the expected size from the deduced amino acid structure of the hUlip gene.

To study the regulation of hULIP proteins, we used a rabbit anti-peptide A serum (18), which is raised against the peptide YDGPVFDLTTTPK (amino acids 499–511; Figs. 3 and 6). To determine whether the anti-peptide serum recognizes the hUlip protein, the *in vitro* translated hULIP protein was reacted with specific anti-peptide A or a control antiserum in the absence or the presence of the immunizing peptide, and the immunoprecipitates were analyzed by SDS-PAGE (Fig. 5A). These results indicate that the mRNA transcripts synthesized from hUlip cDNA translate a 60-kDa protein that is recognized by the anti-peptide A serum and competed out by co-incubation with the immunizing peptide. Immunoprecipitation with a normal rabbit antiserum did not detect hUlip proteins. The smaller peptide fragments synthesized by the rabbit reticulocyte lysate may represent truncated protein products of the hUlip gene, because immunoprecipitation of these truncated proteins by the anti-peptide A serum was also inhibited by the immunizing peptide (Fig. 5A). The hULIP peptide is found in its rodent homologue ULIP but not in the other members of this family (Fig. 6). Western analysis indicated that the anti-peptide A antibody detected several hULIP proteins of 58, 60, 65, and 70 kDa in extracts from RA-treated SMS-KCNR, and the immunodetection of these bands was specifically blocked if the immunizing peptide was included during the antibody incubation step (Fig. 5B). This antibody did not detect other members

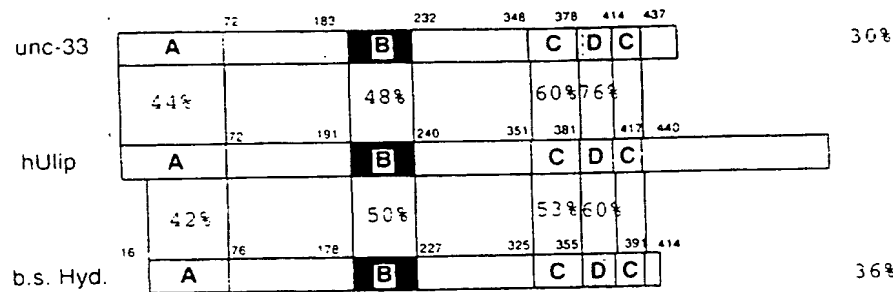


FIG. 4. Definition of highly conserved structural domains in the hULIP protein. Schematic representation of highly conserved domains present in the hULIP protein. Data for this analysis are derived from a protein sequence alignment generated with the GCG-PILEUP software. Sequence accession numbers are: *C. elegans unc-33* mRNA, Z14146; *B. stearothermophilus* D-hydantoinase enzyme, S73773; and *H. sapiens* mRNA for Ulip, Y07818.

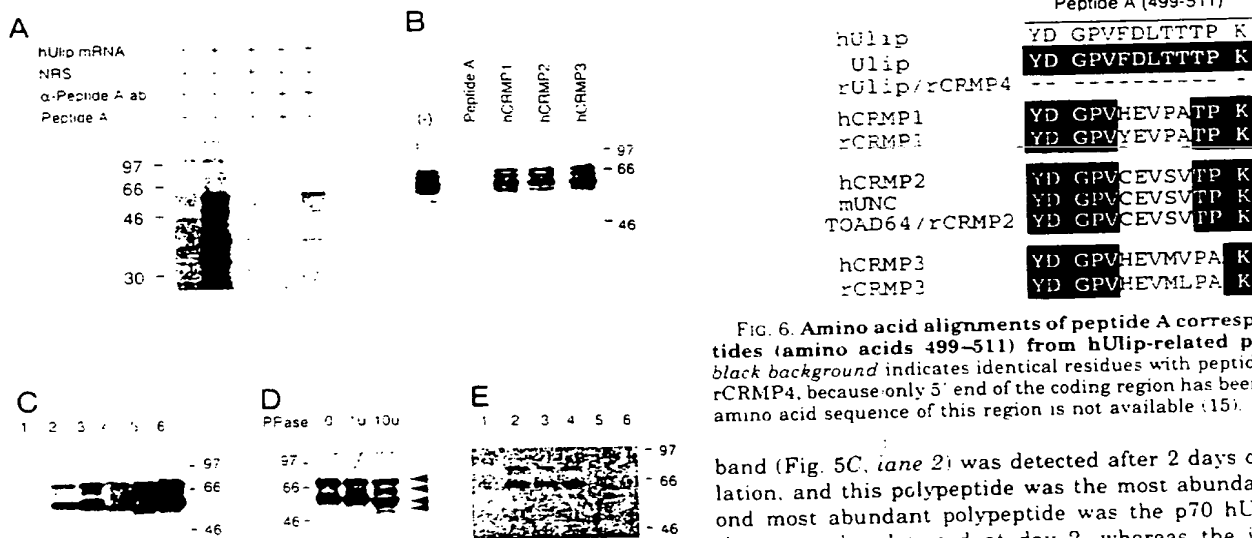


FIG. 5. Expression and regulation of hULIP protein. Western analysis of hULIP protein expression in neuroblastoma cells after treatment with RA. A, recognition by the anti-peptide A serum of the *in vitro* synthesized hULIP protein. *In vitro* translated hULIP protein was either directly mixed with an equal volume of 2 × SDS sample buffer or immunoprecipitated with anti-peptide A serum in the presence or the absence immunogenic peptide and analyzed by SDS-PAGE. B, peptide competition of anti-peptide A serum reactivity on immunoblot. Proteins extracted from SMS-KCNR treated with RA for 6 days were transferred to nitrocellulose filter and probed with anti-peptide A serum in the presence of 200 nM of the immunizing peptide Peptide A or a similar amount of peptide from the corresponding region of the human CRMP1 (rCRMP1), human CRMP2 (or TOAD-64, mUNC, rCRMP2), or human CRMP3 (rCRMP3) genes. C, time course treatment with RA of SMS-KCNR cells. Lane 1, control; lanes 2–6, 2, 4, 6, 9, and 12 days of treatment with 5 μM RA. D, phosphatase treatment of hULIP. 20 μg of protein extract from SMS-KCNR treated with RA for 6 days was incubated with the indicated amount of calf intestinal alkaline phosphatase at 37 °C for 30 min, and then the dephosphorylated forms of hULIP were analyzed by Western blot. Anti-peptide A serum reactive 70-, 65-, 60-, and 58-kDa hULIP proteins were indicated by arrowheads. E, Western blot analysis of hULIP protein expression in different NB cell lines in presence or the absence of RA: SMS-KCNR (lanes 1 and 2), NGP (lanes 3 and 4), and SY5Y (lanes 5 and 6).

of the family because the corresponding peptides (amino acids 499–511) (Fig. 6) from these family members did not block the anti-peptide A antibody (Fig. 5B).

Western analysis was performed on protein extracts isolated from SMS-KCNR cells treated with RA for 2–12 days. Four distinct but immunogenically related polypeptides with apparent electrophoretic mobilities of 70, 65, 60, and 58 kDa were detected (Fig. 5C, lanes 2–6). An increase in the p60 hULIP

FIG. 6. Amino acid alignments of peptide A corresponding peptides (amino acids 499–511) from hULIP-related proteins. The black background indicates identical residues with peptide A. In rUlip/rCRMP4, because only 5' end of the coding region has been reported, its amino acid sequence of this region is not available (15).

band (Fig. 5C, lane 2) was detected after 2 days of RA stimulation, and this polypeptide was the most abundant. The second most abundant polypeptide was the p70 hULIP protein that was also detected at day 2, whereas the intermediate band, p65 hULIP was faintly detected at day 2 (Fig. 5C, lanes 3 and 4, respectively). A fourth protein with an apparent molecular weight of 58 kDa was recognized by the anti-peptide A serum, but it was detected after 9 days of RA treatment. Upon prolonged exposure of the western (Fig. 5B), the 58-kDa hULIP protein could be detected, and this was also competed by the immunizing peptide (data not shown).

The finding that peptide A (499–511 amino acids) specifically inhibits antibody binding to all hULIP proteins (Fig. 5B) indicates that these four proteins all contain an identical antigenic epitope. The p70, p65, and p60 hULIP proteins are detected within 48 h after RA stimulation, the p58 hULIP protein is detected after 9–14 days of RA treatment. The differential regulation of hULIP proteins suggests that they may be translational modifications of a smaller number of proteins. Studies of ULIP, the murine counterpart, indicate that it is a phosphorylated protein (14). To test whether the different proteins detected by the anti-peptide A serum in RA-treated NB cells may be phosphorylated forms of a single protein, protein extracts from 6 day RA-treated KCNR NB were incubated with alkaline phosphatase at 37 °C. After a 30-min incubation, proteins were resolved by SDS-PAGE, blotted, and analyzed with the anti-peptide A serum (Fig. 5D). Western analysis of phosphatase treated extracts resulted in the detection of proteins with apparent molecular masses of 70, 65, 60, and 58 kDa. These studies indicate that the p70 and p60 hULIP proteins may be phosphorylated forms of the p65 and p58 proteins, respectively. The kinetics of detection of the underphosphorylated forms of the proteins suggests that RA may be inducing a specific phosphatase activity.

hULIP protein expression was evaluated in three neuroblastoma cell lines whose differentiation response when treated with RA was variable. In KCNR, RA induced a high level of expression of all three hUlip related proteins in KCNR (Fig. 5E, lane 2). In the NGP NB cell line, the 70- and 60-kDa hULIP proteins are constitutively expressed (Fig. 5E, lane 3), and the level of expression is not significantly altered by RA (Fig. 5E, lane 4). RA is a poor inducer of differentiation in the cell line SY5Y (11), and expression of hULIP proteins in SY5Y (Fig. 5E, lanes 5 and 6) was at a level not detected when compared with the levels of hULIP proteins in KCNR and NGP. Prolonged exposure of blots revealed that RA did induce hULIP in SY5Y (data not shown).

#### DISCUSSION

This paper describes the identification, cloning, and sequence of the human homologue of the mouse Ulip (14) and the rat CRMP4 (15) gene. An analysis of fetal and adult human tissues indicates that hUlip is developmentally regulated like its rodent counterparts. By utilizing RA-induced differentiation of human neuroblastoma cells, we have found that the hUlip gene is transcriptionally regulated during the process of induced differentiation, and there is a dramatic increase in hULIP protein expression during neuritogenesis.

hUlip is a member of a family of evolutionarily conserved and structurally related genes. Homologous sequences were isolated from different species: D-hydantoinase from *P. putida*, *unc-33* from *C. elegans* (16), CRMP62 from chicken (17), the rat gene TOAD-64 (19), the mouse genes Ulip (14) and mUNC<sup>4</sup>, the human genes CRMP1 and CRMP2 (17) several EST sequences (28), and four rat genes CRMP1-4 (15). The presence of multiple sequences with different levels of homology even within the same species suggests that multiple *unc-33* like genes are present among the vertebrates. hUlip has an overall homology with the bacterial enzyme D-hydantoinase and the *C. elegans* *unc-33* of only 36 and 30%, respectively, yet it is possible to detect areas of the proteins that have a higher level of identity in the primary sequence (Fig. 4). A previous study identified at least three areas of homology that may represent functional domains in the protein by comparison of Ulip with the D-hydantoinase of *P. putida* (14). However, by comparing hUlip to the D-hydantoinase of *B. stearothermophilus*, we have detected an additional region D within the C region with a high number of conserved residues present in the human, bacterial, and worm genes that may have functional significance. To date it has not been possible to assign enzymatic functions to these domains in other *unc-33*-like proteins (CRMP62 and Ulip), and studies are in progress to address this issue in hUlip. It may be possible that those conserved areas underlie functional domains of the protein. Such a hypothesis is supported by the evidence that the function of the chicken *unc-33* homologue, CRMP62, as a collapsin response mediator may be suppressed by injecting an antibody raised against a peptide within the first conserved domain A into the cell (17).

Clues to the role these proteins play during differentiation have been inferred from the functional identification of several members of this family: all members share homology with *unc-33*, a *C. elegans* mutant with uncoordinated movements and defects in axonal outgrowth (16); chicken CRMP1 was identified as a mediator of collapsin-induced growth cone retraction (17); and TOAD-64 is one of the earliest and most abundant proteins expressed in post-mitotic neurons during corticogenesis and migration, yet its expression decreases dramatically in adult neural cells (18, 19). Family members have an intracellular cytoplasmic location and the proteins, includ-

ing hUlip, contain consensus sites of phosphorylation for protein kinase C, casein kinase II, protein kinase A, and "proline-directed" kinases as well as *N*-myristoylation sites. This has lead to speculation that these proteins may be intracellular mediators of collapsin signal transduction and play a role in axonal guidance during neurite outgrowth.

The analysis of hUlip distribution in fetal and adult human tissue samples of the central and peripheral nervous system is consistent with the reports for the tissue distribution of the murine homologue of hUlip as well as other members of this gene family in other species (14-19). We found a high level of specific mRNA in fetal brain and spinal cord samples but not in any of the non-neuronal and adult tissues examined. This finding may underlie a tissue-specific regulation of hUlip expression and suggests that, like its rodent counterparts, it has a specific role in developing the human nervous system. Members of this family of genes have been almost exclusively localized in rodent neural tissues by *in situ* analysis (15, 17), although Ulip expression has been found in muscle localized at the neuromuscular junction (14).

The finding of differential expression and regulation of hUlip in neuroblastoma cell lines enables biochemical and functional studies of the hUlip gene and protein to be performed that may be important in determining its functional role during neural development. The expression of hUlip mRNA by Northern blot analysis was increased in RA-treated SMS-KCNR cells after 24-48 h of treatment reaching the peak at 8 days and stabilizing thereafter (Fig. 1A). The increase in hUlip parallels the kinetics of neurite extension that peak 6-8 days after RA treatment (24). This increase in the steady-state levels of hUlip mRNA is due in part to an increase in gene transcription as shown by RUN-ON assays (Fig. 1B), indicating that chromatin changes are required to activate the expression of this gene in differentiating neuroblasts. The kinetics of induction of hUlip transcription increase gradually with time after RA treatment (2-fold at 2 days, 20-fold at 7 days (Fig. 1B), and 22-fold at 14 days (13)), indicating that the effects of RA may be indirect and not mediated by RA receptors. hUlip mRNA expression is also increased in RA-treated NB cells at a time in which cells have arrested in G<sub>1</sub> of the cell cycle and neuritogenesis begins. This is similar to the pattern of expression of TOAD-64 that is absent in the mitotic precursors of corical neurons but is highly expressed once cells have stopped dividing (18, 19). However, constitutive hUlip expression can be detected in the proliferating neuroblastoma cell line, NGP, and this suggests that arrest of cell growth may be coincident and not required for expression of hUlip in neuroblastoma. Conversely, it is possible that during normal development hUlip expression is coordinately regulated with arrest of cell growth, yet this process is disrupted as a consequence of genetic changes leading to tumorigenesis in some neuroblastoma cell lines.

During RA-induced growth arrest and neurite extension in KCNR NB cells, four immunologically related hULIP proteins with apparent molecular masses of 58, 60, 65, and 70 kDa were detected. The specificity of the anti-peptide A antiserum to detect hULIP and not other members of the family indicates that these proteins are post-translational modifications of hULIP. Whether other members of this family are expressed during RA-induced differentiation of NB cells is not known, although it is possible as rat studies have shown that all CRMP family members are differentially and developmentally expressed in the peripheral nervous system (15). We have found that phosphatase treatment of protein lysates from RA-treated cells indicates that the p65 may be an underphosphorylated form of p70, whereas p58 may be an underphosphorylated form of p60 (Fig. 5D). During kinetic analysis, p58 and p65 are

<sup>4</sup> C. Gaetano, T. Matsuo, and C. J. Thiele, unpublished observations.

detected later after RA treatment and in lower abundance compared with p60 and p70, respectively. This suggests that during RA-induced differentiation a phosphatase may be induced. The products of the chicken, rat, and mouse genes, CRMP62, TOAD64, and Ulip, have been shown to be phosphoproteins. In particular the phosphorylation of several proteolytic peptides are altered after nerve growth factor treatment of PC12 cells, indicating a possible involvement of the *unc-33*-like proteins in the Trk-mediated signal transduction pathways (14). Nerve growth factor stimulates an increase in TOAD-64 protein (18) and Ulip phosphorylation in PC12 cells (14). These observations indicate that members of the *unc-33* gene family may also be involved in Trk signal transduction pathway. We find that hUlip expression can be stimulated in the RA-treated NB cell line SY5Y and the addition of BDNF enhances hUlip expression.<sup>4</sup> However, RA is also known to regulate a number of membrane receptors in NB including TrkB (11, 27), epidermal growth factor receptor (13) *c-kit* (29), transforming growth factor- $\beta$  receptors (30), and c-RET (31). Thus it is possible that activation of these or other unidentified signaling pathways may also contribute to the increased transcription of hUlip mRNA in NB cells stimulated by RA.

In this study, we present the isolation and preliminary characterization of the human homologue of the Ulip gene. The ability of RA to regulate transcription of this gene provides a model to study factors affecting the developmental expression of this protein. Furthermore, the variable expression of hUlip in neuroblastoma cells offers a model system to study the function of this protein as it relates to neuronal differentiation.

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# Antibodies to a subpopulation of glial cells and a 66 kDa developmental protein in patients with paraneoplastic neurological syndromes

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## Abstract

**Background**—Paraneoplastic neurological syndromes (PNS) are inflammatory disorders that probably depend on autoimmune processes. Several autoantibodies (anti-Hu, anti-Ri, and anti-Yo) have been characterised in PNS and proved to be helpful in the diagnosis. However, these do not account for all the cases and the possibility that other types of antibodies could be detected was investigated.

**Methods and results**—Of 45 patients with PNS whose serum was probed on paraformaldehyde fixed rat brain sections, 11 patients were identified whose serum samples recognised a cytoplasmic antigen in a subpopulation of glial cells in the white matter of adult rat brainstem, cerebellum, and spinal cord that were double labelled with a monoclonal antibody specific for oligodendrocytes. All serum samples reacted with a 66 kDa protein of newborn rat brain on western blot analysis. These antibodies were designated as anti-CV2 antibodies. Only one of the 11 patients had one of the well characterised autoantibodies (anti-Hu). Five patients had cerebellar degeneration, three had limbic encephalitis, two had encephalomyelitis, and one had Lambert-Eaton myasthenic syndrome. The tumours were small cell lung cancer or undifferentiated mediastinal cancer in seven patients, uterine sarcoma in two, and malignant thymoma in two. Among 1061 control serum samples, only two patients had anti-CV2 antibodies. One had small cell lung cancer and the other malignant thymoma.

**Conclusions**—The detection of anti-CV2 antibodies in patients with neurological disorders should be considered as an indication of the presence of an occult cancer.

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**Keywords:** paraneoplastic neurological syndromes; oligodendrocytes; anti-CV2 autoantibodies

Paraneoplastic neurological syndromes (PNS) are rare inflammatory disorders of the central and peripheral nervous system that possibly depend on autoimmune processes directed against antigens shared between the tumour and the nervous system.<sup>1</sup> Several autoantibodies correlated with the neurological disorders

and the tumours have been characterised in PNS and proved to be helpful in the diagnosis.<sup>2,3</sup> Thus most patients with the encephalomyelitis-sensory neuropathy complex associated with anti-Hu (or ANNA-1) antibodies have small cell lung cancer.<sup>4,5</sup> Those with paraneoplastic cerebellar degeneration and anti-Yo (or PCA-1) antibodies have gynaecological tumours.<sup>6,7</sup> Most of the patients with anti-Ri (or ANNA-2) antibodies and opsoclonus/ataxia have breast cancers.<sup>8</sup> However, these autoantibodies do not account for all patients with PNS.<sup>10-12</sup> Some of the patients negative for these antibodies could actually harbour autoantibodies that could be identified by other methods, in particular, by using fixing procedures which improve antigen preservation.

Recently, by using paraformaldehyde fixed rat brain sections, we described two patients with PNS and antibodies that reacted with a cytoplasmic antigen in a subpopulation of glial cells in the brainstem and cerebellar white matter.<sup>13,14</sup> To investigate the possibility that these antibodies could be relevant for the diagnosis of cancer in patients with neurological disorders suspected to be paraneoplastic, we screened 45 patients with PNS, 128 patients with tumour, and 933 control patients of various types for the presence of these antibodies.

## Materials and methods

### PATIENTS

Serum and CSF from 45 patients with PNS were collected and stored at -20°C until use. Control serum samples were obtained from 900 patients with various inflammatory or non-inflammatory neurological diseases without cancer, 128 patients with cancer and no PNS (37 small cell lung carcinoma, 14 other lung cancer, 25 intestinal adenocarcinoma, two lymphoma, 29 gynaecological cancer (breast and ovary), seven various carcinomas, 14 malignant thymoma with myasthenia gravis) and 33 normal control subjects. Control CSF samples were obtained from 30 patients with various neurological syndromes. As positive controls for the identification of anti-Hu, anti-Ri, and anti-Yo antibodies, we used three serum samples obtained from Dr Josep Dalmau (Memorial Sloan Kettering Cancer Center, New York, USA).

### TISSUE PREPARATION FOR IMMUNOHISTOCHEMISTRY

Adult rat, newborn rat, and day 22 rat embryonic tissues (E22) were obtained from OFA

rats (IFFA CREDO, France). Human brain samples were obtained from a 71 year old woman without neurological disease and from a 24 week embryo after a spontaneous abortion.

Adult rats were anaesthetised with Nembutal and killed by intracardiac perfusion of 4% paraformaldehyde and 0.2% picric acid diluted in phosphate buffer (0.1 M, pH 7.4). Tissues were postfixed for 24 hours at 4°C in the same solution, rinsed in phosphate buffer for 12 hours, cryoprotected in a solution of 25% sucrose in phosphate buffer, rapidly frozen in isopentane chilled at -60°C in liquid nitrogen, and stored at -80°C. Human brain, rat embryo, and newborn rat brain were fixed at 4°C for four days by immersion in 4% paraformaldehyde and 0.2% picric acid diluted in phosphate buffer (0.1 M, pH 7.4) and then cryoprotected as described above.

#### IMMUNOCYTOCHEMISTRY

##### *Immunofluorescence*

To screen the serum samples we used an indirect immunofluorescence technique. Frozen sections (12 µm) were cut on a cryostat, mounted on gelatin-covered slides, treated for two hours in phosphate buffered saline (PBS) and 1% bovine serum albumin (BSA) with 0.1% Triton X 100, and incubated for 12 hours with the patient's serum in PBS-1% BSA at room temperature (diluted 1/100 on adult rat brain sections and 1/1000 on E22 rat embryo sections). After three washes with PBS-1% BSA-0.1% Triton X 100, the sections were incubated for two hours with 1/100 diluted fluorescein-conjugated rabbit anti-human antiserum (Dakopatts, Denmark) in PBS-1% BSA. After washing in PBS, the slides were mounted in moviol and examined with an Axiophot Zeiss microscope. Control sections were incubated with either fluorescein conjugated antihuman IgG antiserum alone, patient's serum alone, or control serum samples and the fluorescein conjugated antibody at the same dilution. Positive serum samples were then tested to establish end point dilution.

##### *Immunoperoxidase*

To confirm the positivity of the serum samples detected by screening with immunofluorescence, we used both indirect and direct immunoperoxidase labelling after biotinylation of the patient's serum IgG.

*Indirect immunoperoxidase method*—Frozen tissue sections fixed by paraformaldehyde were sequentially incubated with 0.3% hydrogen peroxide (to destroy tissue peroxidase activity) and 10% normal rabbit serum (to prevent non-specific binding of rabbit IgG) or 1% BSA. After incubation for 12 hours with patient's serum diluted 1/1000 and washes, the sections were incubated for two hours with biotinylated rabbit antihuman IgG antiserum (Jackson, Baltimore, USA) diluted 1/1000 in PBS-1% BSA. Bound human IgG was visualised by incubation with avidin-biotin-peroxidase (Vectastain ABC complex, Vector) and developed with 0.05% diaminobenzidine tetrahydrochloride (Sigma, St Louis, MO).

Control sections were stained with 15 serum samples from patients without PNS using the same protocol.

*Patient's IgG biotinylation*—IgG was prepared from the serum samples of four patients (3, 6, 7, and 8) and conjugated with biotin as described by Furneaux *et al.*<sup>13</sup> After treatment with hydrogen peroxide and incubation with 10% normal human serum, the sections were incubated with biotinylated IgG (1 µg/ml) for 12 hours at room temperature. Bound human IgG was visualised by incubation with avidin-biotin-peroxidase (Vectastain ABC complex, Vector) as described above. Control sections were obtained with biotinylated IgG from a healthy patient.

##### *Double labelling*

To determine whether the CV2 reactive cells were oligodendrocytes, we used a double labelling with Rip, a mouse monoclonal IgG1 antibody specific for oligodendroglial cells provided by Dr B Zalc (INSERM U 289, Paris, France).<sup>14</sup>

Double labelling was performed on white matter of adult rat spinal cord. Longitudinal sections of spinal cord were incubated overnight at room temperature, simultaneously with anti-CV2 antiserum (diluted 1/300) and with Rip antibody (diluted 1/20). Then, sections were stained with appropriate fluorescein conjugated antiserum (anti-CV2) and Texas red conjugated antiserum (Rip) by the method previously described.

#### BIOCHEMICAL AND IMMUNOCHEMICAL METHODS

##### *Subcellular fractionation of brain*

Samples of fresh rat or postmortem human brain were homogenised with sodium phosphate buffer (5 mM, pH 7) containing protease inhibitors (1/1000 leupeptine, 1/1000 pepstatine, 2.5/1000 aprotinin (Sigma)), and 0.3 M sucrose, using a hand held teflon glass potter homogeniser (clearance 0.1 mm) and centrifuged for 10 minutes at 1000 g to remove nuclei and large tissue fragments. In some experiments, the supernatant (S1) was then centrifuged for one hour at 6000 g. The pellet (P2) contained a crude membrane fraction. The supernatant (S2) was then centrifuged for 30 minutes at 100 000 g to separate soluble proteins (S3) from a crude microsome fraction (P3). The fractions P2, S3, and P3 were then diluted with buffer containing protease inhibitors to adjust the protein concentration to 1 mg/ml and stored at -20°C.

##### *Immunoblotting*

Each fraction was diluted (1:1; v/v) with 250 mM Tris HCl buffer, pH 6.8 containing 2% SDS, 0.01% bromophenol blue, 20 mM dithiothreitol, and 10% glycerol. For each experiment, 18 µg proteins per lane were separated on 12% polyacrylamide gels by electrophoresis and electrically transferred to PVDF membranes (Immobilon membranes, Millipore). Strips of PVDF were first incubated for 30 minutes with 1% BSA and 0.1%



Tween 20 in Tris buffered saline (TBS) to saturate non-specific protein binding sites and then for 12 hours with the patient's serum diluted in the same solution. After three washes with TBS-Tween 20 (0.1% v/v) and 0.5% BSA, bound antibodies were disclosed by incubating the PVDF strips for two hours with biotinylated rabbit antihuman IgG anti-serum (Jackson; Baltimore) diluted 1/1000 in TBS-1% BSA, rinsed for 3 × 15 minutes in TBS, and then incubated for one hour with streptavidin-peroxidase (Jackson, Baltimore) 1/2000 in the same buffer. The colour reaction was developed with diaminobenzidine tetrahydrochloride. Apparent molecular weights were estimated from standards (Pharmacia). The presence of anti-Hu, anti-Ri, and anti-Yo antibodies has been confirmed separately by appropriate methods.<sup>1</sup>

#### Immunoprecipitation

Twenty microlitres of the serum of patients 2 and 5 and of a control serum were incubated for one hour at 4°C with 1 ml S3 fraction containing 1 mg protein. Protein A sepharose (20 mg) was suspended in 200 µl TBS, added to the mixture, and gently agitated at 4°C. After one hour, a cushion of 100 µl 1 M sucrose was applied to the bottom of the reaction tubes which were then centrifuged for five minutes at 10 000 g. The upper fraction was discarded along with most of the sucrose and the pellet containing the protein A sepharose was washed three times in 1 ml TBS and centrifuged at 10 000 g for five minutes. As much of the liquid fraction as possible was removed after each wash using a fine pipette. Finally, the protein A sepharose was resuspended in 100 µl SDS-Page sample buffer containing DTT and incubated for 15 minutes at room temperature. Sucrose (100 ml, 1 M) was added to the bottom of the reaction tube which was then centrifuged for five minutes at 10 000 g.

Denatured eluted proteins were recovered in the upper phase and sepharose in the lower. Samples of the superior phase were then analysed by western blot as described above.

#### Results

Among the 45 serum samples of patients with PNS, 17 did not have detectable antineuronal system antibodies, 11 had antineuronal nuclei antibodies (eight corresponded to anti-Hu antibodies by western blot and none to anti-Ri antibodies), seven had antibodies against Purkinje cells (five corresponded to anti-Yo antibodies by western blot). Furthermore, 11 patients had antibodies that reacted with a cytoplasmic antigen in a subpopulation of glial cells in the white matter according to a highly specific reproducible pattern characteristic of antibodies that we have designated as anti-CV2. Among them, 10 were negative for anti-Hu, anti-Ri, and anti-Yo antibodies and one had anti-Hu antibodies. The same cytoplasmic glial labelling was also found with the CSF available from seven of these 11 patients. Among the 1061 control serum samples (from normal controls, patients with various neurological diseases, and patients with cancers without PNS) only two serum samples labelled cells in the white matter with a pattern identical to that of the 11 aforementioned patients. One of them had metastatic small cell lung cancer and the other malignant thymoma.

#### CLINICAL SYNDROMES

Seven of the 11 patients with PNS and anti-CV2 antibodies were examined by either JH or JCA. The clinical records of the others were obtained from their physicians. Patients 1 and 2 have been reported previously.<sup>13,14</sup> The table summarises the data concerning the clinical symptoms, the results of CSF examination,

Clinical data, CSF study and serum and CSF anti-CV2 titres in the 11 patients with paraneoplastic neurological syndromes

Patient no	Age/sex	Delay* (months)	Neurological syndrome	Tumour	CSF				Antibody titre (immunofluorescence)	
					WCC (mm <sup>3</sup> ) (normal < 2)	Protein (mg/100ml) (normal < 45)	IgG (normal < 17)	oligoclonal bands	Serum	CSF
1	66/F	-24	Cerebellar ataxia Sensory motor neuropathy Uveitis and retinopathy Limbic encephalitis	Undifferentiated carcinoma	40	53	15%	yes	1/15 000	1/300
2	53/F	-1	Limbic encephalitis	Malignant lymphoepithelial thymoma	36	30	ND	ND	1/15 000	1/100
3	49/M	-1	Limbic encephalitis	Small cell lung carcinoma	3	62	ND	ND	1/1000	1/100
4	53/M	+7	Myasthenia gravis Encephalopathy	Malignant lymphoepithelial thymoma	7	30	ND	ND	1/5000	ND
5†	69/M	-6	Frontal dementia Cerebellar ataxia Sensory neuropathy	Small cell lung carcinoma	50	150	20%	No	1/100 000	1/2000
6	70/F	+3	Cerebellar ataxia Sensory neuropathy Loss of vision	Uterine sarcoma	8	38	23%	Yes	1/10 000	1/100
7	60/M	-20	Limbic encephalitis Sensory neuropathy	Small cell lung carcinoma	ND	ND	ND	ND	1/10 000	ND
8	69/M	-5	Cerebellar ataxia Sensory neuropathy	Small cell lung carcinoma	200	200	ND	Yes	1/20 000	1/2000
9‡	61/M	-2	Lambert-Eaton myasthenic syndrome	Small cell lung carcinoma	< 2	90	12%	No	1/10 000	1/1000
10	72/F	-4	Cerebellar ataxia	Cervix uterus sarcoma	ND	ND	ND	ND	1/5000	ND
11	63/M	-1	Cerebellar ataxia	Small cell lung carcinoma	50	30	25%	Yes	1/15000	ND

\*Negative numbers indicate that the neurological syndrome preceded the diagnosis of tumour.

†Associated with an anti-Hu antibody (1/60 000).

‡Associated with an anti-VGCC-antibody.

ND = not done.

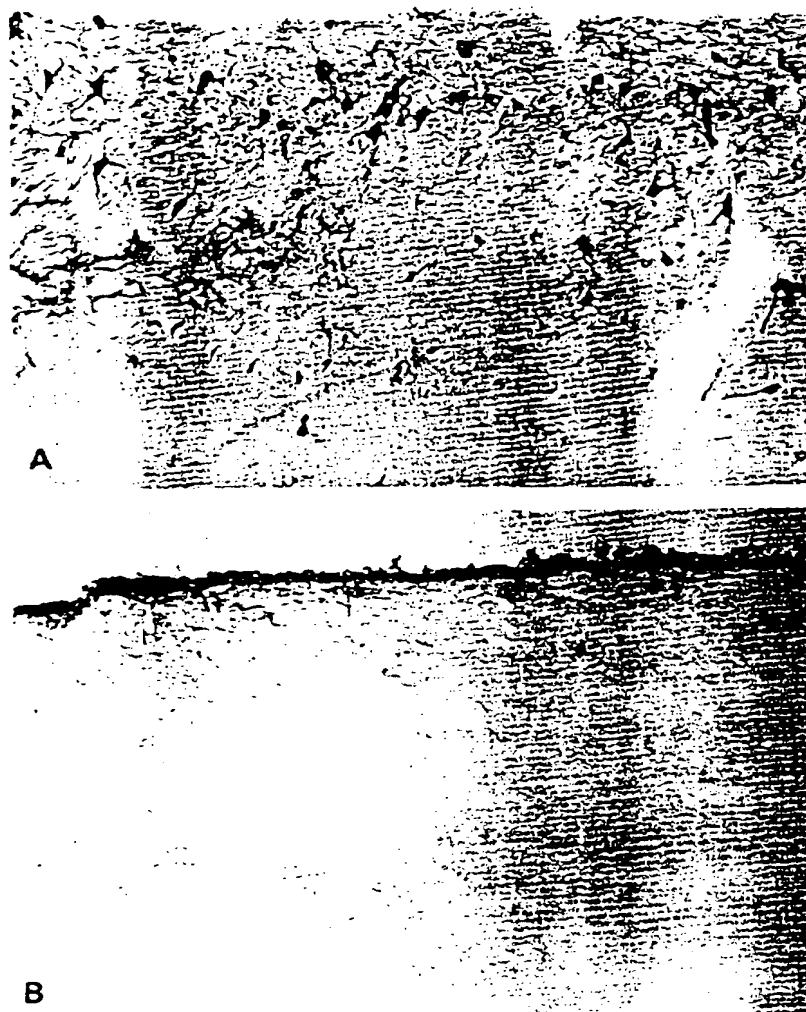


Figure 1 Indirect immunoperoxidase on a six week old rat brainstem (ventral spinocerebellar tract). (A) serum from a patient with anti-CV2 antibodies (patient 1 at 1/1000 dilution) (original magnification  $\times 200$ ). Anti-CV2 antibodies react with the cytoplasm and processes of glial cells in the white matter. (B) serum from a control patient without cancer or neurological disease at 1/100 dilution (original magnification  $\times 200$ ).

and the associated tumours. In eight patients (1, 2, 3, 5, 7, 8, 9, and 11), the neurological syndromes preceded the discovery of the tumour by one to 24 months. In the three patients (4, 6, and 10) in whom neurological symptoms occurred after the diagnosis of the tumour, the tumour was not in remission at that time. Seven patients had small cell lung cancer, two had uterine sarcoma, and two had lymphoepithelial malignant thymoma.

In three patients (2, 3, and 7), the main symptoms consisted of epilepsy and recent memory impairment which were highly suggestive of limbic encephalitis. In two of them (patients 2 and 3), brain MRI showed hippocampal high signals on T2 weighted sequences. Five patients (1, 6, 8, 10, 11), had a prominent severe cerebellar ataxia. Two of them also had uveitis or retinopathy (patients 1 and 6). Patient 4 rapidly became bedridden due to a severe encephalopathy with epilepsy, mutism, apraxia, and stiffness. Patient 5 had dementia, facial dyskinesia, and cerebellar ataxia. The last patient (9) had Lambert-

Eaton myasthenic syndrome only. In addition to symptoms of CNS dysfunction, sensory or sensory-motor peripheral neuropathy was present in five patients (1, 5, 6, 7, and 8).

Samples were obtained at necropsy for three patients (1, 5, and 8). Two patients (1 and 8) had severe Purkinje cell loss with slight but widespread inflammatory cell reaction and one (patient 5) showed a diffuse inflammatory encephalomyelitis. In these three patients, no primary demyelinating lesions were found in the brain.

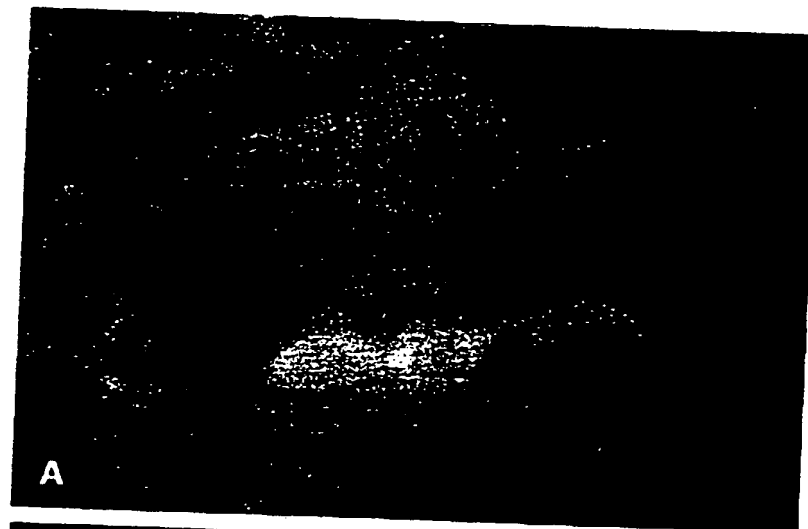
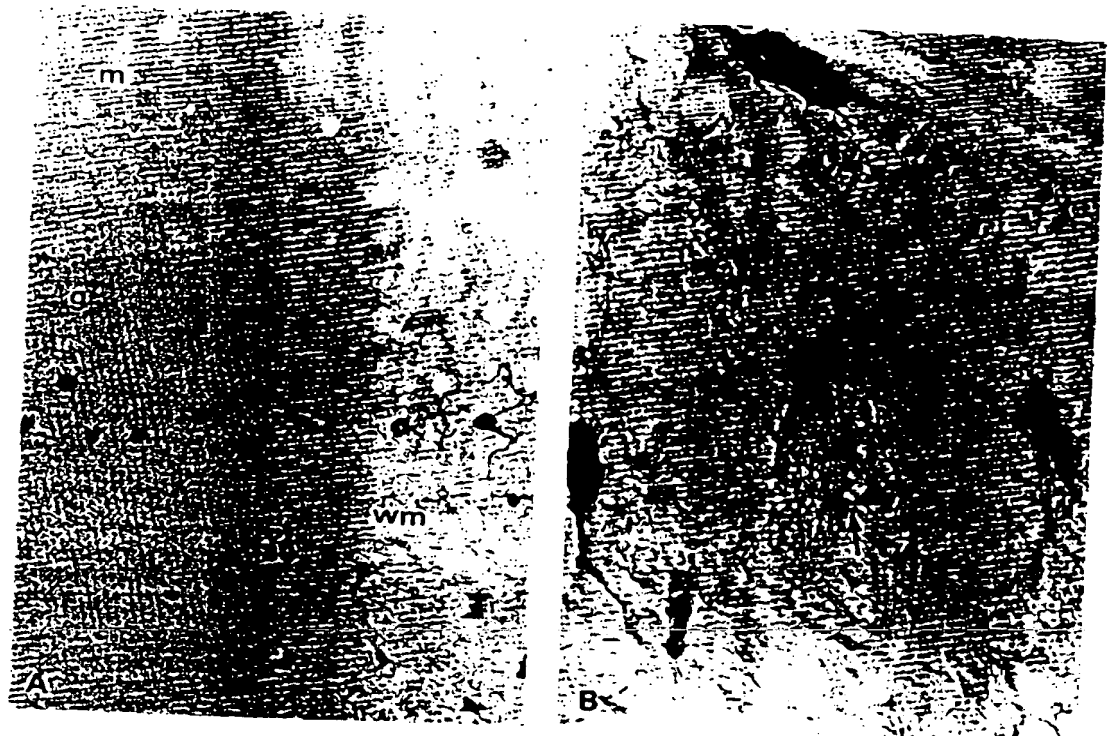
#### IMMUNOHISTOCHEMISTRY ON RAT AND HUMAN BRAIN

On adult rat brain, serum samples and CSF gave identical patterns by indirect immunofluorescence and immunoperoxidase methods. Anti-CV2 antibodies labelled the cytoplasm of cells in the white matter and their processes that sometimes surrounded myelinated fibres homogeneously (figs 1 and 2). Cell nuclei were always negative. The morphology of labelled cells and their clustering like strings of beads along myelinated tracts suggested that they were oligodendrocytes. Double labelling showed that cells positive with anti-CV2 antibodies were also stained with Rip, a mouse monoclonal antibody specific for oligodendrocytes (fig 3),<sup>16</sup> confirming this hypothesis.

Cells stained with anti-CV2 antibodies were numerous in the spinal tract of the trigeminal nerve, the pyramidal tract, and the medial longitudinal fasciculus of the brainstem (fig 4). Immunolabelled cells were also numerous in the cerebellar peduncles and cerebellar white matter, and in all the spinal cord tracts. Labelling obtained with the IgG fraction of four serum samples after biotinylation was identical (fig 2). No labelling of oligodendrocytes occurred in the corpus callosum and cerebral cortex.

The pattern of staining obtained with anti-CV2 antiserum was clearly different from that with anti-Hu antibodies which labelled the nuclei of neurons and at low dilution the nuclei of glial cells (data not shown). By immunofluorescence, the limiting dilution of anti-CV2 serum antibodies from the 11 patients with PNS and the two positive patients with cancer and no PNS ranged from 1/1000 to 1/100 000. The anti-CV2 antibody titre from the seven CSF samples available ranged from 1/100 to 1/2000. In the patient who also had anti-Hu antibodies (patient 5), serum anti-Hu antibody titre was 1/60 000 by our immunohistochemistry technique on rat brain whereas it was 1/100 000 for anti-CV2 antibodies. In addition, four out of the 11 serum samples of patients with anti-CV2 antibodies and PNS labelled cells in the subgranular layer of the dentate gyrus of the hippocampus at 1/1000 to 1/10000 dilution (patient 1, 2, 7, and 11). At low dilution (1/100) some serum samples with anti-CV2 antibodies labelled the cytoplasm of neurons in the thalamus, the cortex, or the hippocampus whereas no labelling was seen on adult rat liver, kidney, testes, ovary, spleen, and adrenal gland.

**Figure 2** (A) Immunoperoxidase on a six week old rat cerebellum with biotinylated IgG of patient 7 (1 µg/ml) (original magnification  $\times 400$ ). m = Molecular layer; g = granular layer; wm = white matter. (B): Immunoperoxidase on a six week old rat brainstem (spinal tract of the trigeminal nerve) with biotinylated IgG of patient 7 (1 µg/ml) ( $\times 1000$ ). Some positive processes surround an axon (arrow) suggesting that these cells are oligodendrocytes.



**Figure 3** Longitudinal section of white matter of the rat spinal cord ( $\times 1000$ ). (A) Interfascicular cells visualised by indirect immunostaining with Rip monoclonal mouse antibody. (B) The same section double labelled with the serum of a patient containing anti-CV2 antibodies (serum from patient 7, diluted 1/300).

In developing rat embryo, from embryonic day 12 to birth all the anti-CV2 serum samples specifically labelled cells and their processes in postmitotic regions of both the central and peripheral nervous system (figs 5A and 6). Neither proliferating cells in the ventricular zones and the outer granular layer of the cerebellum nor non-neural tissues were stained. By contrast, anti-Hu antibodies only labelled cell bodies in the same regions (fig 5C). Serum from 30 control patients gave no staining at 1/100 dilution (fig 5B). After birth, the staining progressively decreased to a pattern identical to that in adult brain.

We tested seven serum samples of the 11 patients with PNS and anti-CV2 antibodies (patients 1 to 7) by indirect immunoperoxidase on adult human brain. Four serum samples (from patients 1, 2, 5, and 7) which stained rat brain at the highest dilutions, labelled glial cells in the white matter of the adult human brainstem and cerebellum (at a dilution of 1/1000) with a pattern similar to that in the rat brain (fig 7). The other serum samples were negative as were the 30 control samples.

#### IMMUNOBLOT

Each serum sample was tested on different cellular fractions of adult and newborn rat brain. A principal band of 66 kD apparent molecular weight was recognised at 1/100 dilution by all the 11 serum samples and the seven available CSF samples of patients with PNS and the serum samples of the two positive patients with cancer and no PNS. This protein was particularly enriched in the S3 subcellular fraction of newborn rat brain. Thus this fraction was used to estimate the titre of the serum samples. Among all serum samples only two (patients 6 and 10) were negative at 1/500 dilution (fig 8) and only three serum samples

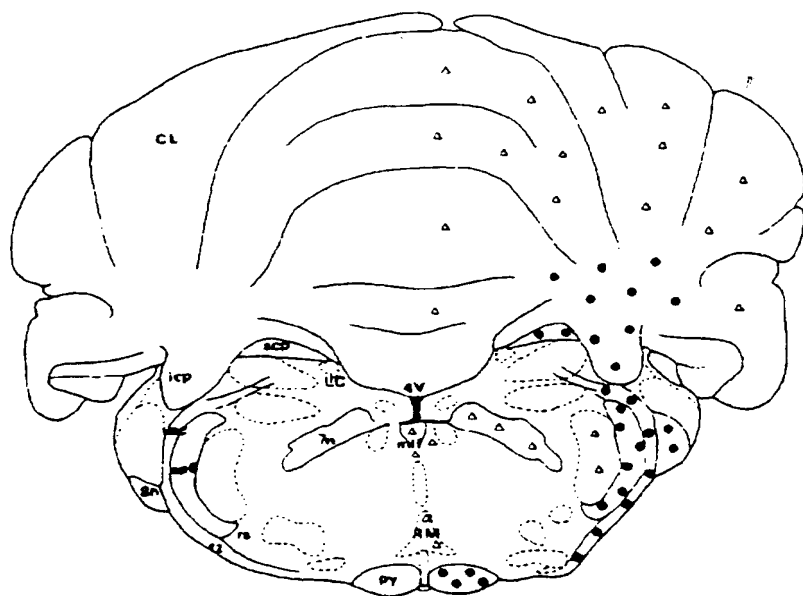


Figure 4 Schematic distribution of CV2 positive cells on a section of rat brainstem. ● High density of CV2 positive cells and △ low density of CV2 positive cells. 4V = Fourth ventricle; 7n = facial nerve; 8n = vestibulocochlear nerve; CL = cerebellar lobules; icp = inferior cerebellar peduncle; LC = locus coeruleus; mlf = medial longitudinal fasciculus; py = pyramidal tract; RM = raphe magnus nuclei; rs = rubrospinal tract; scp = superior cerebellar peduncle; sp5 = spinal tract trigeminal nerve; tz = trapezoid body; vsc = ventral spinocerebellar tract.

remained positive at 1/1000 dilution (patients 2, 5, and the patient with small cell lung cancer and no PNS). The 66 kDa band was not stained by any of the serum samples in the S3 fraction of rat liver. None of the 150 control serum samples (diluted at 1/100) reacted with a 66 kDa band from the S3 fraction of newborn rat brain.

Western blots of the S3 soluble fraction of adult rat brain showed lower levels of expression of the 66 kDa protein than in newborn rat brain and three serum samples gave no staining (patients 6, 8, and 10).

In the S3 fraction of human embryo brain, nine of the 11 serum samples of patients with PNS and the serum samples of the two positive patients with cancer and no PNS labelled a 66 kDa band at 1/100 and 1/500 dilutions. Patients 6 and 10 were negative. With the S3 fraction of adult human brain, six serum samples (patients 2, 4, 5, 7, 9, and 11) reacted with a 66 kDa band with variable staining intensities whereas the other serum samples were negative.

#### IMMUNOPRECIPITATION

To test whether these serum samples recognised the same antigen, we immunoprecipitated the S3 soluble fraction of newborn rat

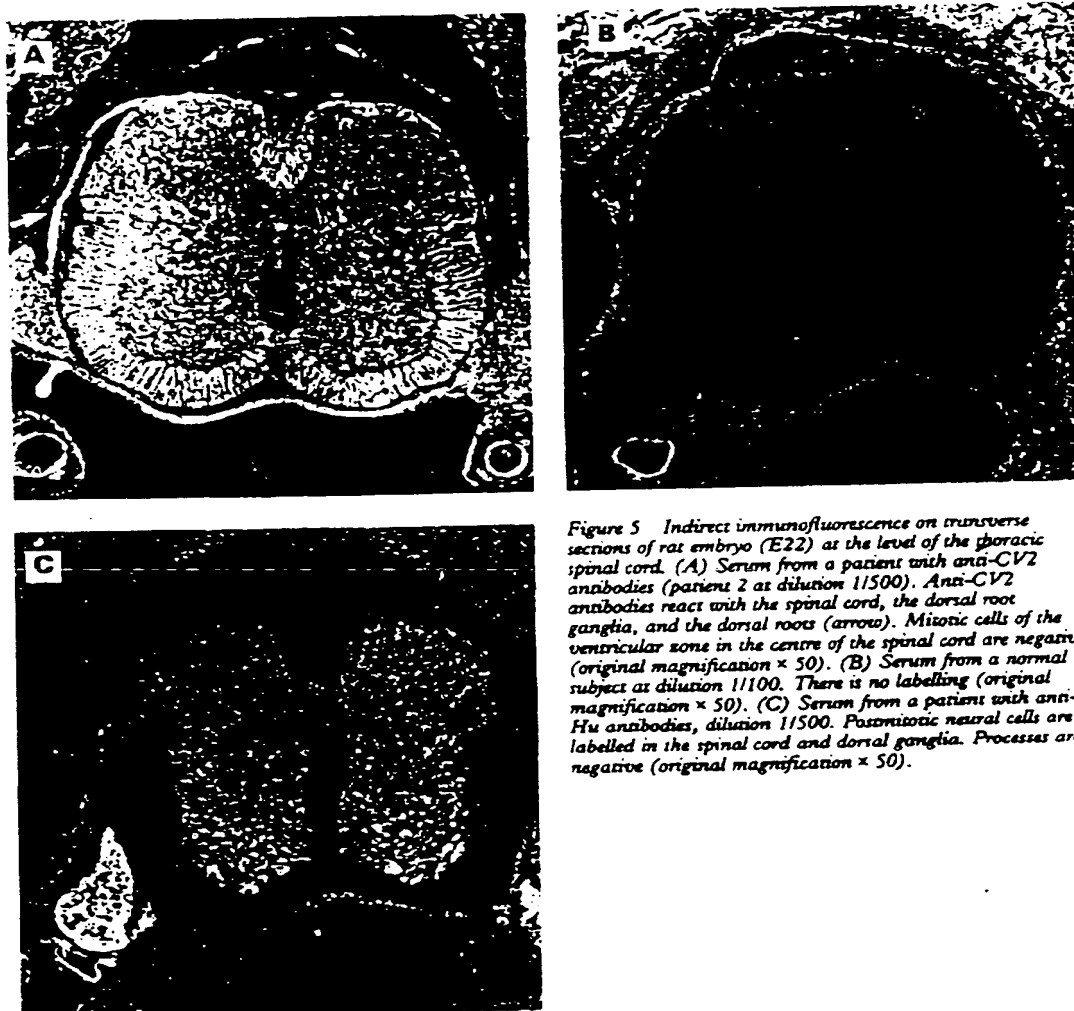


Figure 5 Indirect immunofluorescence on transverse sections of rat embryo (E22) at the level of the thoracic spinal cord. (A) Serum from a patient with anti-CV2 antibodies (patient 2 at dilution 1/500). Anti-CV2 antibodies react with the spinal cord, the dorsal root ganglia, and the dorsal roots (arrow). Mitotic cells of the ventricular zone in the centre of the spinal cord are negative (original magnification  $\times 50$ ). (B) Serum from a normal subject at dilution 1/100. There is no labelling (original magnification  $\times 50$ ). (C) Serum from a patient with anti-Hu antibodies, dilution 1/500. Postmitotic neural cells are labelled in the spinal cord and dorsal ganglia. Processes are negative (original magnification  $\times 50$ ).

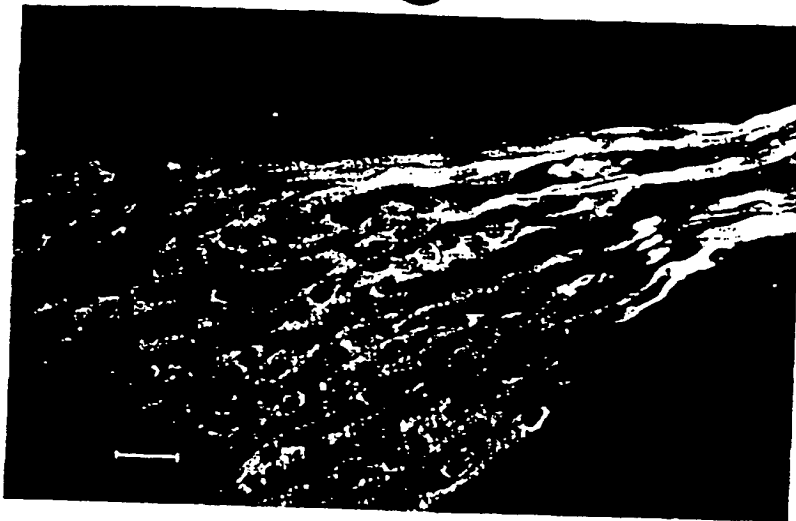


Figure 6 Dorsal root ganglion and peripheral nerve axons in E22 rat embryo stained with serum from a patient with anti-CV2 antibodies (patient 1 diluted 1/1500). Cell bodies and processes are labelled (bar = 30  $\mu$ m).

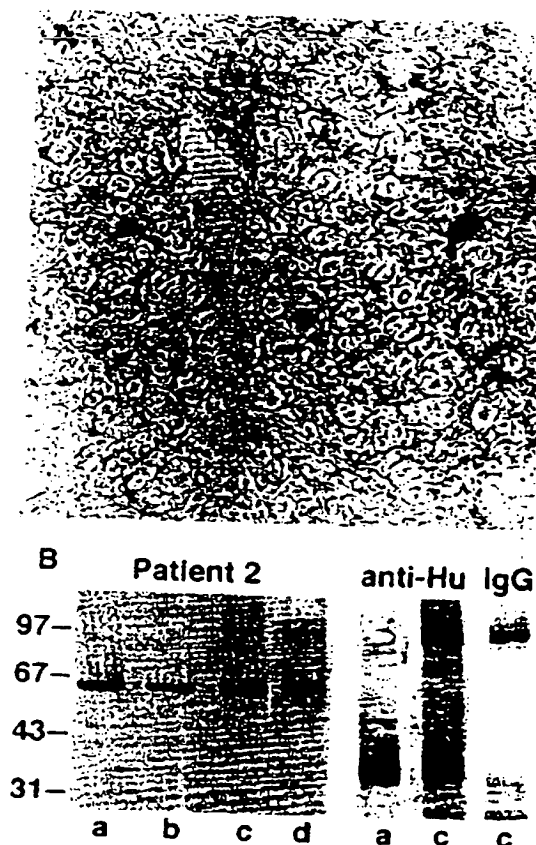


Figure 7 (A) Indirect immunoperoxidase on human brainstem (pyramidal tract) with the serum from a patient with anti-CV2 antibodies (patient 2) at 1/11000 dilution. Glial cells are stained. Some positive processes surround an axon (arrow) suggesting that positive cells are oligodendrocytes (original magnification  $\times 400$ ). (B) Western blots with three different serum samples (6  $\mu$ g protein per lane): serum from a patient with anti-CV2 antibodies diluted 1/1500 (patient 2), serum from a patient with anti-Hu antibodies diluted 1/1500 (anti-Hu) and an anti-human IgG rabbit antiserum diluted 1/1000 (IgG). a = S3 fraction of new born rat brain; b = S3 fraction of adult rat brain; c = S3 fraction of human embryonic brain (week 24); d = S3 fraction of adult human brainstem. The serum of patient 2 recognised a 66 kDa band in both human and rat brain extracts. The serum with anti-Hu antibodies and the anti-human IgG rabbit antiserum are negative for the 66 kDa band. Bands of about 97 kDa on human extracts correspond to endogenous IgGs.

brain with the serum samples from patients 2 and 5. On the immunoprecipitated extracts, the seven anti-CV2 antiserum samples tested (from patients 1 to 7) recognised the 66 kDa band (weakly for serum 6; fig 9) and the band was not recognised in either of the two serum samples of normal control patients. No band was recognised by anti-CV2 serum samples in the extracts immunoprecipitated with the control serum.

### Discussion

The identification of the well characterised autoantibodies—anti-Hu (ANNA-1), anti-Ri (ANNA-2), and anti-Yo (PCA-1)—associated with PNS relies on immunohistochemical and western blot methods.<sup>14</sup> Immunohistochemistry is usually performed on acetone fixed sections from human or rodent brain and only high serum dilutions are considered.<sup>17</sup> For western blot, appropriate antigen enriched preparations of purified neurons or fusion proteins are used and developed at high serum dilutions.<sup>1</sup> However, many patients with PNS have no detectable anti-Hu, anti-Ri, or anti-Yo antibodies.<sup>10-12</sup> Although the presence of other antineuronal cell antibodies has been reported using these procedures, they usually concern individual patients.<sup>18-21</sup> and many patients with PNS have no detectable autoantibodies. Among these antibody negative patients, the PNS could depend on a non-immunological process or could be mediated by cellular immunity. However, it cannot be excluded that antibody negative patients harbour antineuronal system autoantibodies not shown by usual procedures—for example, anti-VGCC autoantibodies associated with myasthenic Lambert-Eaton syndromes and small cell lung cancer cannot be shown by standard immunohistochemistry and western blot.<sup>22</sup>

To improve antigen preservation more than with acetone postfixed sections, we tried to detect antibodies by using sections from rat brain fixed by perfusion with paraformaldehyde. With this technique, we identified a subgroup of patients with PNS, negative for anti-Hu, anti-Ri, or anti-Yo antibodies, whose serum samples labelled a particular subpopulation of glial cells in adult rat brain which we have designated as anti-CV2. For several reasons this pattern of staining cannot be confused with non-specific glial cell labelling reported at low dilution with normal control serum samples or serum samples from patients with anti-Hu antibodies.<sup>1</sup> Firstly, anti-CV2 antibodies only stained a subpopulation of glial cells specifically distributed in the white matter of the cerebellum, brainstem, and spinal cord. These were probably oligodendrocytes as shown by double labelling with the Rip monoclonal antibody. Secondly, except in two patients with cancer, we never saw a similar pattern of staining in 1061 control serum samples, even at low serum dilution (1/100). Thirdly, the staining obtained with anti-CV2 antibodies was always found with high serum dilution, ranging from 1/1000 to 1/100 000.

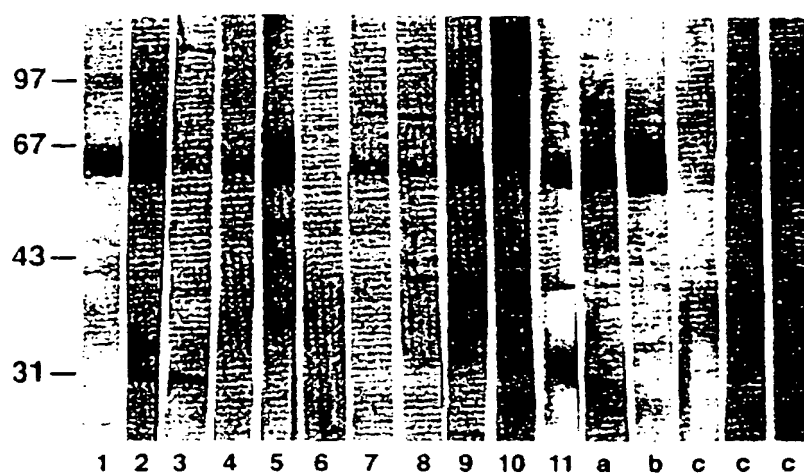


Figure 8 Western blots of 18  $\mu$ g protein per lane of the S3 soluble fraction of new born rat brain incubated with serum samples (dilution 1/1500) and disclosed by immunoperoxidase. 1 to 11 = Serum samples from patients with anti-CV2 antibodies and PNS. a and b = serum samples from the two patients, one with small cell lung carcinoma (a) and the other (b) with malignant thymoma who showed anti-CV2 antibodies by immunohistochemistry. c = Control patients with neurological syndromes and no cancer. All the serum samples with the pattern of anti-CV2 antibodies by immunohistochemistry label a band of apparent molecular weight 66 kDa, except patients 6 and 10 who had uterine sarcoma. These two serum samples were positive at 1/1100 serum dilution (data not shown).

Fixation procedures were crucial for the detection of anti-CV2 antibodies, because we found no labelling with acetone fixed sections of rat brain<sup>13</sup> and obtained poor results with less stringent fixation procedures (data not shown). These results could explain the variability of labelling on human brain sections, probably caused by postmortem loss of the antigen or to poor antigen preservation with postfixation. Similar difficulties occur with serum samples from patients with Hodgkin's disease and cerebellar degeneration. In these patients antibodies against Purkinje cells are sometimes more easily detected with mouse brain than with human brain.<sup>23</sup> However, despite variability in staining with the different serum samples, our results show that the CV2 antigen is present in human brain.

### Immunoprecipitation

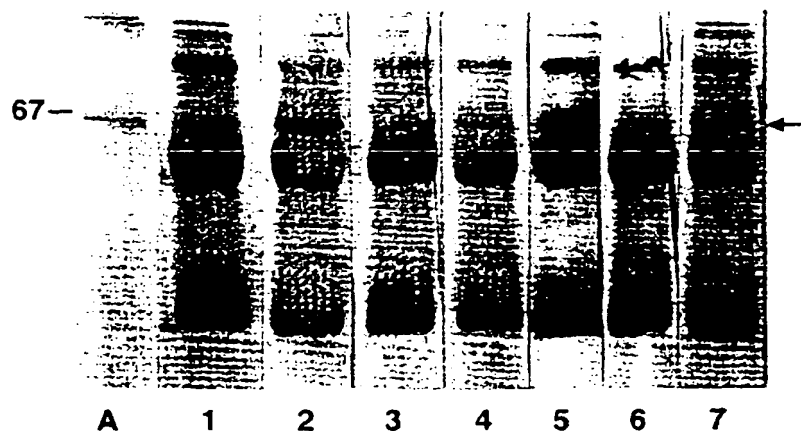


Figure 9 S3 fraction of new born rat brain was immunoprecipitated with the serum of patient 2. The immunoprecipitate was processed for western blot analysis and incubated with the serum samples of seven patients with anti-CV2 antibodies (lane 1 to 7) at 1/1100 dilution. All of them recognise the 66 kD band (arrow). Other bands correspond to denatured immunoglobulins in the precipitate. Lane A is a western blot with the S3 fraction of newborn rat brain incubated with the serum of patient 2.

The progressive restriction of the labelling from a widespread staining of postmitotic neural cells in rat embryo to a subpopulation of glial cells in the adult suggests that CV2 antigen is ontologically down regulated. This regulation could explain the differences of the immunoreactivity by western blot between newborn and adult brain. Indeed, if all anti-CV2 serum samples recognised a 66 kDa band on newborn rat brain extract, the immunoreactivity was different between serum samples with adult brain. Thus we used S3 subcellular fractions of new born rat brain for western blot. However, the high sensitivity obtained by immunohistochemistry contrast with the lower sensitivity on western blot. On silver stained electrophoresis gels with newborn rat brain extract, the 66 kDa protein corresponds to a faint band suggesting that the amount of the antigen in the brain is small (data not shown). This suggests that this protein could be present in a low quantity in the brain which could explain the low sensitivity of western blot. Obviously, further work is needed to purify the 66 kDa protein for routine western blots with higher serum dilution. The availability of a recombinant protein, currently in progress, will probably improve the sensibility of western blots.

Anti-CV2 antibodies are strongly associated with cancer, predominantly in patients with PNS. However, they do not seem to be specific for one type of neurological disorder nor of one type of tumour. Indeed, although anti-CV2 antibodies seem to be mainly associated with small cell lung cancer, they occur in several cases with uterine sarcoma or malignant thymoma and a range of neurological syndromes including Lambert-Eaton myasthenic syndrome, limbic encephalitis, encephalomyelitis, and cerebellar degeneration. However, all of these neurological syndromes are known to be paraneoplastic.<sup>1</sup>

One of the criteria proposed for the characterisation of antibodies associated with PNS is that these antibodies should react with neurons in regions corresponding to the neurological syndrome.<sup>17</sup> This presupposes that the antibodies are the primary mediator of the immune attack. For the moment, this hypothesis needs confirmation.<sup>2</sup> Indeed, although some data suggest that anti-Hu antibodies could provoke lysis and subsequently neuronal death,<sup>24</sup> other studies failed to confirm this hypothesis.<sup>25</sup> Alternatively, the antibodies may be only part of a complex autoimmune reaction involving several tumour and brain antigens and several effectors of humoral and cellular immunity.<sup>26</sup> Thus a polyclonal activation of B cells could lead to the production of antibodies that are not directly involved in the autoimmune process.<sup>4</sup> Antistriated muscle antibodies are a good example of this phenomenon in patients with myasthenia gravis and thymoma.<sup>27</sup> Whether anti-CV2 antibodies result from a polyclonal activation or are directly involved in the PNS remains to be established. Anti-CV2 antibodies react, in adult brain, with a subpopulation of oligodendrocytes, a cell class that is not considered to

be the usual target of the immune attack in PNS. This suggests that anti-CV2 antibodies could result from an incidental immunological reaction. In addition, one patient had both anti-Hu and anti-CV2 antibodies, arguing in favour of a polyclonal activation.

However, whatever the physiopathological relevance of anti-CV2 antibodies, their detection is valuable in the diagnosis of PNS. Indeed, 10 out of the 11 patients with PNS and anti-CV2 antibodies were negative for anti-Hu, anti-Ri, or anti-Yo antibodies. In four cases investigated before the discovery of the tumour, the detection of anti-CV2 antibodies predicted the presence of a cancer with the high probability that it was a small cell lung cancer. This was confirmed in each case by the discovery of mediastinal lymph nodes on chest CT. If a chest CT is negative for thymoma or metastatic lymph nodes, patients with anti-CV2 antibodies should be preferentially investigated for uterine sarcoma.

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# Identification and Molecular Characterization of Unc-33-Like Phosphoprotein (Ulip), a Putative Mammalian Homolog of the Axonal Guidance-Associated *unc-33* Gene Product

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The control of neuritic extension and guidance is critical for the development, maturation, and regeneration of functional neuronal circuits. We identified a neuronal 64–85 kDa phosphoprotein, the expression of which in mouse brain is regulated during development, reaching a peak at ~5 d postnatal, when maturation of neurons and synaptic connections is highly active. The amino acid sequence of the mouse protein deduced from its cloned cDNA reveals similarities with that of the neuritic outgrowth- and guidance-related product of the *unc-33* gene in *Caenorhabditis elegans*. The regulation of

its phosphorylation in response to nerve growth factor, as well as its localization in neurites and growth cones and at the neuromuscular junction, further indicates that Ulip (for Unc-33-like phosphoprotein) is not only a structural but likely is also a functional mammalian homolog of Unc-33, potentially involved in the control of neuritic outgrowth and axonal guidance.

**Key words:** neuronal development; axonal guidance; neuritic elongation; Ulip; *unc-33*; hydantoinases; toad-64; *crmp-62*; phosphoprotein; PC12 cells; neuromuscular junction

The development of the nervous system requires, after proliferation and migration of neuronal precursor cells, the growth of neuritic processes and their guidance toward the appropriate targets with which they will establish synaptic contacts. Several intracellular proteins, the expression or regulation of which could be related closely to neuritic growth and its control, have been characterized (for review, see Skene, 1989). Genetic screening of mutants impaired in the proper targeting of neuronal circuits in invertebrates such as *Caenorhabditis elegans* (White et al., 1986) yielded a large set of mutants exhibiting abnormal, uncoordinated (*unc* mutation) movements (Brenner, 1974; Swanson et al., 1984). Aside from mutations in genes coding for muscle proteins, proteins implicated in migration of precursor cells, neurotransmitter release, and synapse formation, several mutations resulting in axonal outgrowth defects were identified (Hedgecock et al., 1985; Desai et al., 1988; Siddiqui and Culotti, 1991; McIntire et al., 1994). Some of these correspond to extracellular guidance molecules such as *unc-6* (Desai et al., 1988; McIntire et al., 1994) for which netrins have been identified recently as their mammalian homologs (Serafini et al., 1994), whereas others like *unc-33*

(Li et al., 1992) or *unc-44* correspond to intracellular, often cytoskeleton-associated or related proteins (Otsuka et al., 1995).

In our own search for proteins involved in the relay and integration of intracellular signals triggered by extracellular regulatory factors, we identified stathmin (Sobel, 1991), a small, ubiquitous, and cytosolic phosphoprotein that is highly expressed in neurons of the developing brain (Chneiweiss et al., 1989; Koppel et al., 1990) and is phosphorylated in response to growth and differentiation factors such as nerve growth factor (NGF) (Doye et al., 1990). It is the generic member of a highly conserved protein family (Maucuer et al., 1993) that includes the neuronal differentiation-related protein SCG10 (Anderson and Axel, 1985; Stein et al., 1988).

We have observed previously that a rabbit polyclonal antibody originally directed against an internal peptide of stathmin also recognized a 64 kDa brain-specific phosphoprotein (Koppel et al., 1990). This protein is highly expressed in the neonatal mouse and is downregulated in the adult, therefore appearing to be implicated potentially in the development of the nervous system. We report in this study the further molecular purification and characterization of this phosphoprotein, as well as the molecular cloning of its cDNA. The corresponding amino acid sequence reveals similarities with that of a bacterial hydantoinase and with the neuronal development-related gene product of *unc-33* in *C. elegans*; we therefore designate the mouse protein as Ulip (for Unc-33-like phosphoprotein). Mutations in the *unc-33* gene were shown to result in neuritic outgrowth and guidance defects (Hedgecock et al., 1985; Hedgecock et al., 1987; Desai et al., 1988). Ulip appears also to be part of a Unc-33-related protein family, another member of which is represented by the toad-64 protein identified recently in rat (Minturn et al., 1995) and the chick *crmp-62* protein (Goshima et al., 1995), which was published after submission of this paper and was proposed to be involved in the intracellular response to collapse. Together with the molecular properties of the mouse Ulip protein, our present characterization of its biological expression and regulation in neuronal cells

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suggests that it is most likely a functional mammalian homolog of the *unc-33* gene product, which suggests further that it participates in the control of neurite outgrowth and axonal guidance during the development and regeneration of the mammalian nervous system.

## MATERIALS AND METHODS

### Materials

Swiss mice were from the local breeding facility. Frozen brain samples from postmortem humans were provided by Dr. F. Javoy-Agid (INSERM U289, Paris, France). DEAE-Sephacrose Cl-6B, phenyl-Sepharose Cl-4B, nucleic acid purification (NAP)-25 columns, an electrophoresis molecular weight kit, an isoelectric focusing calibration kit, and ampholines were from Pharmacia (Uppsala, Sweden). The dT-primed Uni-Zap cDNA library from whole neonatal mouse brain and the helper phage kit were from Stratagene (La Jolla, CA). The prokaryotic pET19 vector expression kit was from Novagen (Madison, WI). The Sequenase Version 2.0 DNA-sequencing kit was from United States Biochemical (Cleveland, OH). PC12 cells were purchased from the American Type Culture Collection (Rockville, MD), culture media and fetal calf serum were from Gibco (Epagny, France), and horse serum was from Serovial (Vogelgrun, France). Other materials were obtained as follows. Aprotinin, pepstatin, leupeptin, urea, dithiothreitol, Trizma, agarose, isopropylthiogalactoside (IPTG), and phenol were from Sigma (Deisenhofen, Germany). Restriction enzymes, T4-DNA ligase, and polynucleotide kinase were from New England Biolabs (Beverly, MA). Calf intestine alkaline phosphatase and NGF were from Boehringer Mannheim (Mannheim, Germany). Reagents for polyacrylamide gels, serum albumin, and casein were from Serva (Heidelberg, Germany). Ammonium sulfate, silver nitrate, and other standard reagents were from Prolabo (Paris, France). SDS was from Fluka (St. Quentin-Fallavier, France). [ $\gamma$ - $^{32}$ P]ATP,  $^{125}$ I-labeled protein A, the Megaprime DNA-labeling system, and electrogenerated chemiluminescence (ECL) Western blotting reagents were from Amersham (Buckinghamshire, UK).

### Methods

**Partial purification of Ulip.** Partially purified Ulip was obtained from neonatal mouse brains through three purification steps. Neonatal mouse brains (800; 70 gm) were homogenized with a Polytron homogenizer in 4 vol of homogenization buffer (25 mM Na phosphate, pH 7.8, 1 mM EGTA, 10  $\mu$ g/ml leupeptin, 25  $\mu$ g/ml aprotinin, and 10  $\mu$ g/ml pepstatin). Homogenates were centrifuged for 10 min at 4000  $\times$  g. Pellets were resuspended in 2 vol of homogenization buffer, homogenized, and centrifuged as before. The supernatants of the two centrifugations were assembled, sonicated, and centrifuged for 1 hr at 100,000  $\times$  g. The supernatant (S2) was loaded on a DEAE-Sephacrose Cl-6B column (1.75 cm $^2$   $\times$  26 cm) equilibrated with 100 ml of buffer A (25 mM Na phosphate, pH 7.8, 1 mM EGTA) at a flow rate of 30 ml/hr. Proteins were eluted in 300 ml of a linear gradient of 0–250 mM NaCl in buffer A, and 5 ml samples were collected. The Ulip-containing fractions were pooled, and solid ammonium sulfate was added up to 20% saturation. This pool was loaded on a phenyl-Sepharose Cl-4B column (1.75 cm $^2$   $\times$  22 cm) that was equilibrated previously with 100 ml of buffer B (10 mM Na phosphate, pH 7.8, 1 mM EGTA) containing 20% saturated ammonium sulfate. The proteins were eluted in a decreasing linear gradient from 20 to 0% saturated ammonium sulfate in buffer B. The Ulip-containing fractions were pooled and dialyzed twice against 20 vol of buffer A. The proteins were concentrated on a small (10 ml) DEAE-Sephacrose Cl-6B column and eluted with 400 mM NaCl in buffer A. The eluate was desalted on a Sephadex G-25 (NAP-10) column and concentrated further to a final volume of 0.5 ml by evaporation. In the final purification step, the concentrated fraction was chromatographed in three successive runs on two serially connected Superose 12 fast protein liquid chromatography (FPLC) columns in buffer C (50 mM Na phosphate, pH 7.2, 150 mM NaCl) at a flow rate of 0.3 ml/min. Fractions (0.6 ml) were collected, and Ulip-enriched fractions were pooled and used for further analysis. The presence of Ulip at the successive purification stages was tested by one-dimensional Western blot using the cross-reactive anti-stathmin antibody. Proteins were quantified using the method of Bradford (1976).

**PAGE.** One-dimensional electrophoresis was performed on 13% polyacrylamide gels according to Laemmli (1970). Two-dimensional PAGE was performed as described previously (Sobel and Tashjian, Jr., 1983). The isoelectric focusing gels contained 2% total ampholines, pH 6–8 and

3–10 at a ratio of 4:1. The second dimension was run on 10% acrylamide gels. Proteins were either immunoblotted (see below) or silver-stained as described previously (Sobel et al., 1989).

**Western blot analysis.** Proteins were transferred from gels onto nitrocellulose in a semidry electroblotting apparatus; maximal recovery of Ulip was achieved in 48 mM Tris and 39 mM glycine buffer containing 5% methanol, instead of 20%, for optimal stathmin transfer. The membrane was saturated with casein (2.5%) in immunoblot solution (12 mM Tris-HCl, pH 7.4, 160 mM NaCl, 0.1% Triton X-100) and probed with either an antiserum against peptide I of rat stathmin (1:10,000) (Koppel et al., 1990) or an antiserum against recombinant Ulip (1:20,000) diluted in immunoblot solution containing 1% casein. Bound antibodies were detected either with  $^{125}$ I-labeled protein A and autoradiography or with peroxidase-bound anti-rabbit antibodies and the ECL kit (Amersham).

**Protein sequence analysis.** Ulip-enriched fractions were separated on two-dimensional polyacrylamide gels. Gels were fixed for 30 min in 25% ethanol and 10% acetic acid and stained for 3 min in 0.1% amidoblack in 1% acetic acid and 40% methanol. Gels were destained in 1% acetic acid, and the spots corresponding to the major form of Ulip were cut out from three gels, pooled, and digested with 2 mg/ml endoprotease Lys C. The peptides eluted from the gel then were separated by HPLC on a DEAE-C18 column with a gradient of 0–55% acetonitrile in 0.1% trifluoroacetic acid. Five clearly separated peptides (I–V) were microsequenced by automatic Edman degradation.

**cDNA library screening.** Molecular genetic procedures were performed according to standard protocols (Sambrook et al., 1989). Two degenerate oligonucleotides corresponding respectively to peptides V and IV were synthesized (Genset, Paris, France): 5'-TC(T/C)TCIACIC(C/A/G)TTIG-TIC(C/T/C)TCIGGIATGICGT-3' (Oli-1); and 5'-TTIC(C/A/G)TCIG-CCCA(T/C)TCIC(G/T)CCA(T/C)TT-3' (Oli-2). Phages ( $7.5 \times 10^5$ ) from a Uni-Zap neonatal mouse brain cDNA library were screened with the two  $^{32}$ P-end-labeled oligonucleotides ( $\sim 2.5 \times 10^6$  cpm/pmol). The final washes were performed in 2 $\times$  SSC (1 $\times$  SSC: 150 mM NaCl, 15 mM Na citrate, pH 7) containing 0.1% SDS at 60°C (Oli-1) and 55°C (Oli-2). Primary screening with Oli-1 identified no clearly positive clone, whereas 14 positive clones were identified with Oli-2 (frequency,  $\sim 1/5000$  clones).

**Nucleotide sequencing.** The Bluescript plasmid vector containing the *EcoRI* and *XhoI* inserts was excised from the phage genome using the ExAssist helper phage (Stratagene) and was introduced into the *SoI*R strain of *Escherichia coli* as described by the manufacturer (Stratagene). Twelve clones with inserts from 1300 to 2100 bp were analyzed further. Restriction analysis and sequencing from the two ends showed that these clones differed only in the length of their 5' ends. *AluI* fragments of the longest insert were subcloned into Bluescript and were sequenced fully by the Sanger dideoxy-termination method (Sanger et al., 1977) using T7 and T3 complementary primers and a modified T7 DNA polymerase (Sequenase). Eight additional Ulip-specific oligonucleotides were used to verify the order and the orientation of the subcloned fragments in the entire cDNA insert.

**Bacterial expression of recombinant Ulip.** An *NdeI* restriction site was introduced into the cDNA coding for Ulip by PCR at the putative ATG translation start site: this was carried out using an oligonucleotide containing the mutated sequence (AATCGCCTATGTCCTACCAGGG-CAAGAAG) and an oligonucleotide 3' to the *AarII* site (TAAG-GCATCTGGAAGTGGGT). The mutated PCR product and the original *AarII*-*XhoI* fragment of the Ulip cDNA were ligated into the *NdeI*-*XhoI*-digested pET19 vector. Nova-Blue bacteria (Novagen) were transformed, and ampicillin-resistant strains were selected for Ulip cDNA inserts and sequenced to verify ligation sites and the PCR product. A BL-21 *E. coli* strain then was transformed with Ulip cDNA-containing vectors, and expression was induced with 5 mM IPTG. Almost all of the His-tagged Ulip protein produced was found in nonsoluble inclusion bodies. The protein was dissolved in a buffer containing 6 M urea, and the protein was purified further on a His-Bind column as described by the manufacturer (Novagen).

**Northern blot analysis.** Total RNAs were isolated from several tissues as described previously (Taylor et al., 1986). RNAs were separated on an agarose gel containing formaldehyde (Sambrook et al., 1989), transferred to a Hybond N membrane (Amersham) in 20 $\times$  SSC, and stained with 2% methylene blue. The blots were probed with a multi-prime-labeled ( $\sim 1.5 \times 10^6$  cpm/ $\mu$ g DNA) *EcoRV*-*XhoI* fragment of the Ulip cDNA. Final washes were performed in 0.1 $\times$  SSPE (1 $\times$  SSPE: 150 mM NaCl, 9 mM Na phosphate, 1 mM EDTA) and 0.1% SDS at 60°C.

**Antiserum production.** Rabbits were immunized with 1 mg of synthetic peptide V (Neosystem, Strasbourg, France) coupled to keyhole limpet hemocyanin or with 0.1 mg of recombinant Ulip in complete Freund's

adjuvant and boosted 4 weeks later with half the amount of peptide (protein) in incomplete Freund's adjuvant.

**Cell culture.** PC12 cells were grown in RPMI 1640 supplemented with 10% horse serum, 5% fetal calf serum, 2 mM glutamine, and 5  $\mu$ g/ml gentamycin. Cells were passaged using 0.025% trypsin and 1 mM EDTA in phosphate-buffered saline (PBS). For induction of differentiation, 20 ng/ml 2.5S NGF was added to the cultures. Differentiation usually was induced during 3 d. For two-dimensional PAGE analysis, cells were scraped from the culture dishes in PBS. They were centrifuged for 10 min at 1500  $\times$  g and sonicated in 3 vol of homogenization buffer containing 50 mM Tris-HCl, pH 7.2, instead of Na phosphate.

**Immunofluorescence procedures.** PC12 cells were fixed in methanol at  $-20^{\circ}\text{C}$  (6 min) and treated with blocking solution containing 3% bovine serum albumin (BSA) in PBS (3 times, 10 min each). The cells were incubated (1 hr) with the antibodies diluted in the blocking buffer and washed with PBS containing 0.1% Tween 20. Fluorescein-conjugated goat anti-rabbit antibodies (Tago, Burlingame, CA) were used to reveal anti-Ulip antibodies, and rhodamine-conjugated antibodies (Bioss, Compiègne, France) were used to reveal anti-neurofilament (68 kDa) or anti-tubulin antibodies (Sigma).

Striatal neurons from 20 d gestational rat embryos were cultured for 8 d and then fixed in 2% *p*-formaldehyde in PBS containing 1 mM  $\text{Ca}^{2+}$  for 30 min at  $4^{\circ}\text{C}$ . This was followed by another incubation in 0.1% glycine and 0.1% Triton X-100 in PBS for 30 min at room temperature. Incubations with antibodies were performed as for PC12 cells.

Control immunolabeling experiments were performed using either preimmune serum or serum preincubated with the recombinant Ulip antigen (10  $\mu$ g protein/ $\mu$ l serum).

Immunostaining procedures on muscle tissue were performed as described previously (Cifuentes-Diaz et al., 1994). Briefly, frozen adult muscles were cut at  $-28^{\circ}\text{C}$ . Cryostat sections (6  $\mu$ m) were incubated directly for 1 hr with the anti-Ulip antiserum diluted 1:500 in blocking solution, washed (1 hr) in PBS containing 0.1% Tween 20, and incubated with fluorescein-conjugated anti-rabbit antibodies (dilution 1:100). After washing, preparations were treated with rhodamine-conjugated  $\alpha$ -bungarotoxin (1:1000, Molecular Probes, Eugene, OR).

For immunostaining of whole-mount teased fibers, small bundles of fixed (1% *p*-formaldehyde) muscle fibers were incubated first with 0.1 M glycine in PBS (30 min) and then with 3% BSA and 0.5% Triton X-100 in PBS (30 min). They were incubated next with anti-Ulip antiserum (1:500) overnight at  $4^{\circ}\text{C}$ , washed, and incubated with secondary antibody. The specificity of the staining was assessed by the omission of the primary antibody or by the use of preimmune serum. Preparations were observed with a conventional fluorescence microscope or with a confocal laser scanning microscope (MRC-600, Bio Rad, Hercules, CA) mounted on an Optiphot II Nikon microscope as described previously (Mège et al., 1992).

## RESULTS

### Ulip is a phosphoprotein present in mouse brain as at least two different isoforms

Two-dimensional Western blot analysis of the soluble fraction of newborn mouse brains showed that an antiserum raised against a peptide corresponding to stathmin residues 15–27 recognized, in addition to the characteristic stathmin spots in the region of 19–20 kDa, three spots corresponding to a more basic protein with an  $M_r$  of  $\sim 64$  kDa (Fig. 1A) (Koppel et al., 1990). This protein, originally designated P60 and which we propose to name Ulip (see introductory remarks), was found in the high-speed supernatant of a mouse brain extract but, unlike stathmin, it was precipitated after boiling.

The molecular characterization of Ulip based on its identification with the anti-stathmin antiserum allowed the production of two rabbit polyclonal sera specific for this protein: (1) an antiserum raised against synthetic peptide V corresponding to the sequence of an internal peptide of Ulip; and (2) an antiserum raised against a recombinant protein produced in bacteria. As shown in Figure 1B, both antisera recognized the Ulip protein in the brain and showed no cross-reactivity with stathmin. Because of its higher sensitivity and specificity, the anti-recombinant Ulip antiserum was used primarily for further investigations. Neverthe-

less, the results obtained with this antiserum always could be reproduced qualitatively with the antiserum raised against the synthetic peptide.

All three available antisera recognized several spots corresponding to various molecular forms of Ulip on two-dimensional immunoblots of a neonatal mouse brain-soluble extract (Fig. 1C). Two groups of spots were recognized by the various antisera in brain extracts: group A, with an  $M_r$  of  $\sim 64$  kDa, and group B, with an  $M_r$  of  $\sim 70$  kDa. The anti-stathmin antiserum detected three increasingly acidic forms within group A, with isoelectric pH (pI) values of 7.1, 6.9, and 6.8 (spots 3, 4b, and 5, respectively). In addition, the anti-Ulip antiserum allowed the detection of additional spots in group A (spots 1, 2, 4a, and 6), as well as spots in group B. The protein forms of both groups migrated with a similar pI pattern, differing only in spots 1 and 6, which could not be detected in group B, and in spot 4, which appeared as a doublet (a and b) in group A, whereas only spot 4a could be detected in group B.

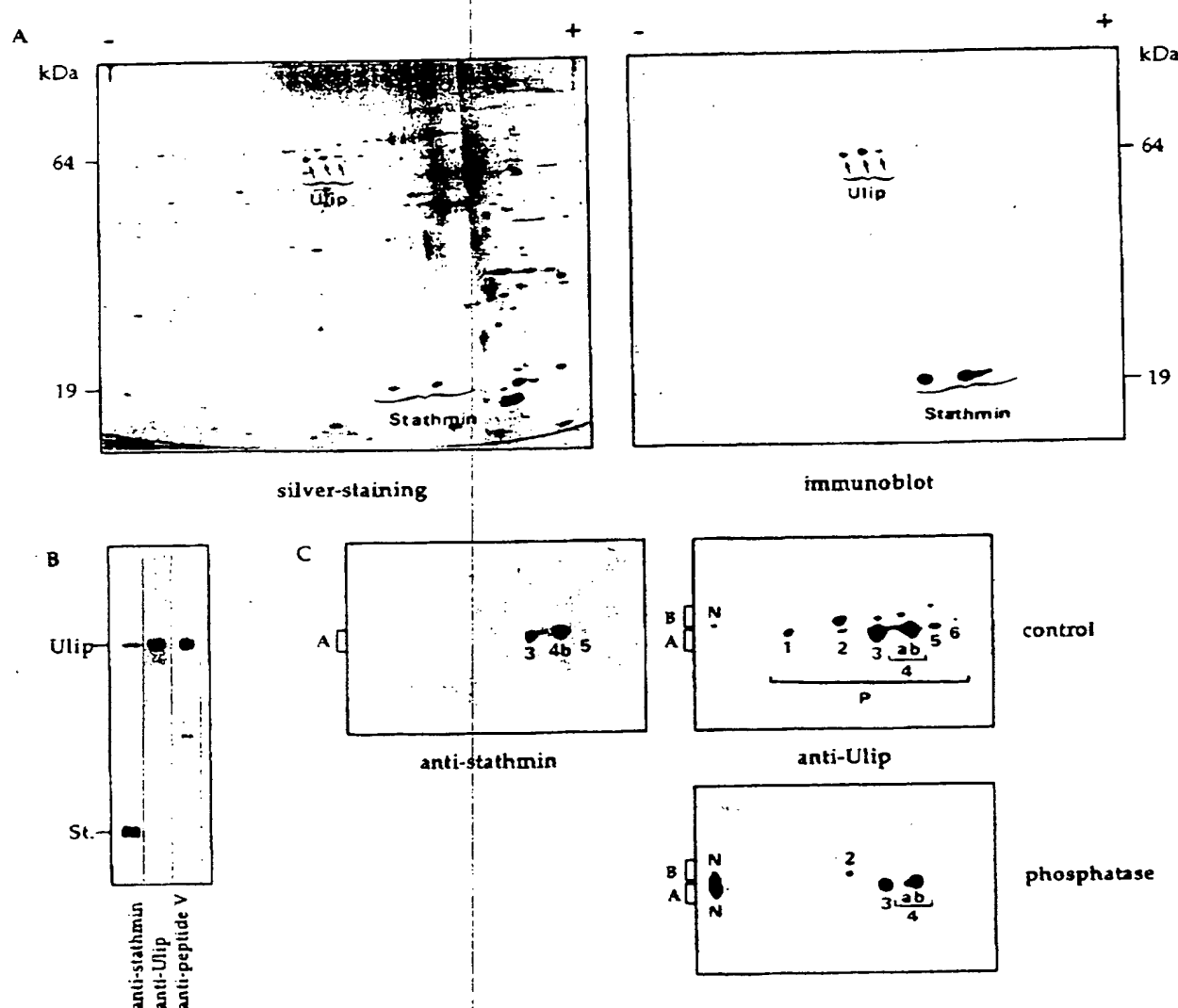
Spots 1–6 migrated at isoelectric points differing by  $\sim 0.1$  pH unit from each other, corresponding approximately to the charge brought by the addition of a phosphate group. The difference between the isoelectric points of A4a and A4b is too small to be explained by an additional phosphate group and, therefore, corresponds more likely to another type of post-translational modification.

The acidic forms 1–6 of both groups A and B could be converted mostly to two more basic forms (pI  $\sim 7.4$ ), designated A<sub>N</sub> and B<sub>N</sub>, respectively, after treatment with alkaline phosphatase for 10 min at  $37^{\circ}\text{C}$  (Fig. 1C). Furthermore, the *in vitro* phosphorylation of the partially purified fraction of Ulip with protein kinase A (PKA), casein kinase II, or cdc2 kinase produced radioactive spots corresponding to forms A1–A5 (data not shown). The acidic forms 1–6, therefore, correspond to increasingly phosphorylated states of the corresponding nonphosphorylated forms N. Together, these observations indicate that groups A and B correspond most likely to the various unphosphorylated (N) and phosphorylated (1–6) states of two isoforms of Ulip. Of interest, a third isoform (C) with an  $M_r$  of  $\sim 85$  kDa was recognized by all three antisera in the rat PC12 pheochromocytoma cell line, as described below (see Fig. 6).

Because the B isoform is not recognized by the anti-stathmin antibody, whereas it is detected with both the anti-peptide V and the anti-recombinant Ulip antisera, it lacks the stathmin-related epitope. This could be the result of a post-translational modification of alternative splicing, or of the expression of a second, closely related gene. Because the lower  $M_r$  form is recognized by the anti-stathmin antiserum, one can exclude the possibility that isoform A is a result of a proteolytic degradation of isoform B. In agreement with this, the recombinant Ulip protein, which contained an additional 20 amino acid "His" tag (see Materials and Methods), migrated only slightly above the A isoform and clearly below the B isoform on SDS gels (data not shown).

### Partial purification of Ulip and cloning and sequencing of a corresponding cDNA

Partial purification of Ulip was achieved from neonatal mouse brains, where it is most abundant. Brains were homogenized, and a high-speed supernatant was prepared and chromatographed on a DEAE-Sephacolumn (see Materials and Methods). Ulip eluted in a single peak between 110 and 220 mM NaCl. It then was chromatographed further on a phenyl-Sephacolumn in a decreasing linear gradient from 12 to 2% ammonium sulfate



**Figure 1.** *A*, Two-dimensional PAGE and immunoblot analysis of a mouse-brain-soluble fraction. A soluble protein extract from neonatal mouse brains (40  $\mu$ g of protein) was electrophoresed on two-dimensional gels (pH 6–8, 13% polyacrylamide) and either silver-stained (*left*) or blotted onto nitrocellulose and subjected to immunodetection with an anti-stathmin antiserum (1:10,000) (*right*). *Stathmin* and *Ulip* spots are indicated. *B*, Characterization of specific anti-Ulip antisera. Antisera were raised specifically against Ulip by injecting rabbits either with peptide V (see Fig. 2) or with the recombinant Ulip protein. The soluble fraction from neonatal mouse brains (15  $\mu$ g of protein) was electrophoresed on a 13% polyacrylamide gel and the immunoblotted with anti-stathmin (1:10,000 dilution), anti-recombinant Ulip (1:20,000), or anti-peptide V (1:2,000) antiserum. Note that the antiserum immunoblotted with anti-stathmin is the most specific (see *Ulip*); *St.*, stathmin. *C*, Multiple forms and *in vitro* dephosphorylation of Ulip. Protein (40  $\mu$ g) from the soluble fraction of a neonatal mouse brain homogenate was separated by two-dimensional PAGE (pH 6–8, 10% acrylamide), and the Ulip protein was revealed with the indicated antisera (same dilutions as in *B*). Two groups of forms with respective  $M_r$  of ~64 kDa (group *A*) and ~70 kDa (group *B*) are indicated, as are the corresponding nonphosphorylated (*N*) and phosphorylated (*P*) forms. The phosphorylated forms are numbered, most likely according to their degree of phosphorylation. The form number 4 seems to be subdivided further in *a* and *b*, differing probably by some other post-translational modification (see text). All of the forms recognized by the anti-recombinant Ulip also were recognized by the anti-peptide V antiserum (data not shown). *Control*, untreated brain homogenate; *phosphatase*, 20 U of alkaline phosphatase was added to the protein fraction and incubated at 37°C for 10 min, resulting in the conversion of most phosphorylated forms of Ulip toward their unphosphorylated states  $N_A$  and  $N_B$ .

saturation. After desalting and concentration, this peak was purified further by molecular sieve, FPLC chromatography on Superose 12HR. The resulting Ulip fraction was enriched ~100-fold compared with the original soluble fraction of the neonatal mouse brain (data not shown).

The enriched Ulip-fraction proteins were separated by two-dimensional PAGE, the A3 spot of Ulip was cut out, and the sequences of five internal peptides were determined (Fig. 2). Two degenerate oligonucleotides (Oli-1 and Oli-2) based on the respective sequences of peptides V and IV were used to screen

750,000 clones of a neonatal mouse brain Uni-Zap cDNA library at high stringency. Oli-1 yielded no clearly positive clones whereas Oli-2 detected 14 positive clones, all of which contained 1500- to 2000-bp-long inserts that differed only by the length of their 5' ends.

The longest sequence identified is presented in Figure 2. It displays an open reading frame (ORF) with a translation initiation consensus sequence (Kozak, 1987) upstream of an initial ATG. The ORF codes for a protein of 570 amino acids, which contain the sequences of all five peptides that were sequenced earlier.

gaattcggcagcagcgaagaaagaaaaataacaaaaaagaaaccccaagccggcgctgaacgcccagtcggaggaggtcgcagctggcg 94  
ggggggcgggtcggggcgaaggggactggggagatttactattgtctctgcagccgcccggggagcccgggggggcgaggcgaagggagggcg 190  
gcacgaccggccggcgccaccattcgctccacctgatctggggcgctgtgcgctgagccagggcgcgagcagcagcagtagcagcagggcagacat 286

ccccctgcagccagaatcgccacc ATG TCC TAC CAG GGC AAG AAG AAC ATT CCG CCG ATC ACG AGT GAC GGT CTT CTA 363  
M S Y Q G K K N I P R I T S D R L L 18

ATC AAG GGA GGG AGA ATC GTC AAT GAT GAT CAG TCC TTT TAT GCT GAT ATT TAC ATG CAG GAT GGC TTA ATA 435  
I K G G R I V N D D Q S F Y A<sub>1</sub> D I Y M E D G L I 42

AAG CAA ATT GCA GAC AAT CTG ATT GTC CCC GGA GGT GTG AAG ACC ATC GAG GCC AAC GGG AAG ATG GTG ATC 507  
K Q I G D N L I V P G G V K T I E A N G K M V I 66

CCT GGA GGC ATT GAC GTC CAT ACC CAC TTC CAG ATG CCT TAC AAG GGA ATG ACC ACA GTG GAC GAT TTC TTC 579  
P G<sub>II</sub> C I D V H<sub>1</sub> T H F Q M P Y K G M [REDACTED] F F 90

CAA GGC ACA AAG GCT GCC TTA GCG GGA GGC ACC ACC ATG ATC ATT GAC CAT GTG GTA CCT GAA CCT GAG TCT 651  
Q G T K A A L A G G T T H I<sub>III</sub> I D H V V P E P E<sub>1</sub> S 114

AGC CTG ACA GAG GCC TAT GAA AAG TGG CGT GAG TGG GCC GAT GGG AAG ACC TGC TGT GAC TAT GCT TTG CAT 723  
S L T E A Y E K W R E<sub>IV</sub> W A D G K<sub>1</sub> S C C D Y A L H 138

GTG GAC ATC ACC CAC TGG AAT GAC AGC CTC AAG CAG GAG GTA CAG AGC CTC AGC AAG GAA AAA GGC GTT AAC 795  
V D I T H W N D S V K Q E V Q S L S K E K G V N 162

TGC TTC ATG GTT TAC ATG GCC TAC AAG GAT TTA TAT CAA GTG TCC AAC ACA GAG CTC TAT GAG ATC TTC ACC 867  
S F M V Y M A Y K D L Y Q V S N T E L Y E I F T 186

TGC CTG GGA GAG CTG GGG GCC ATT GCT CAA GTT CAT GCC GAG AAT GGA GAT ATC ATT GCC CAG GAG CAG GCA 939  
C L G E L G A I A Q V H A E N G D I I A Q E Q A 210

CGG ATG CTG GAA ATG GGG ATA ACC GGC CCA GAA GGC CAC GTT CTG ACC AGA CCG GAA GAG CTG GAA GCT GAG 1011  
R M L E M C I T G P E G H V L S<sub>1</sub> [REDACTED] L E A E 234

GCT GTG TTC CGT GCC ATC ACC GTC GCC AGC CAG ACC AAC TGC CCC CTC TAT GTC ACC AAG GTC ATG ACC AAG 1083  
A V F R A I T V A S Q T N C P L Y V T K V M S K 258

AGC GCG GCT GAT CTC ATC TCA CAA GCC ACG AAG AAA GGA AAT GTG GTC TTT GGC GAG CCC ATC ACT GCC AGC 1155  
S A A D L I S Q A R K K G N V V F G E P I T A S 282

CTG GGA ATA GAT GGA ACT CAT TAC TGG AGT AAG AAC TGG GCC AAA GCA GCT GCG TTT GTG ACA TCC CCA CCT 1227  
L G I D G T H Y W S K N W A K A A A F V T S P [REDACTED] 306

CTG AGC CCT GAC CCT ACC ACA CCT GAC TAC ATC AAC TCC TTG CTG GCC AGC GGG GAT CTG CAG CTC TCT GGA 1299  
[REDACTED] D [REDACTED] D Y I N S L L A S G D L Q L S G 330

AGT GCC CAC TGT ACC TTT AGC ACT GCC CAG AAA GCC ATT GGG AAG GAC AAC TTC ACA GCC ATC CCT GAA GGC 1371  
S A H C T F S T A Q K A I G K D N F T A I P E G 354

ACC AAT GGC GTG GAG GAG CGT ATG TCT GTC ATC TGG GAC AAG GCT GTG GCC ACC GGG AAG ATG GAT GAA AAC 1443  
T N G V E E R<sub>1</sub> M<sub>1</sub> S<sub>1</sub> V I<sub>1</sub> W D K A V A T G K M D E N 378

CAG TTT GTG GCC GTG ACA AGC ACC AAT GCT GCC AAG ATA TTC AAC CTG TAC CCT CGA AAG GGG AGA ATA GCT 1515  
Q F V A V T S T N A A K I F N L Y P R K G R I A 402

GTG GGC TCT GAC AGC GAC CTT GTC ATC TGG GAT CCA GAT GCC TTG AAG ATT GTC TCT GCC AAG AAC CAC CAG 1587  
V G S D S D L V I W D P D A L X I V S A K N H Q 426

TGG GTT GCC GAA TAC AAC ATC TTT GAA GGG ATG GAG CTG CGT GGT GCA CCT CTG GTG GTT ATC TGC CAG GGC 1659  
S V A E Y N I F E G H E L R G A P L V V I C Q G 450

AAG ATC ATG CTG GAA GAT GGC AAC CTG CAC GTG ACC CAG GGG GCT GGC GGC TTC ATT CCC TGC AGC CCA TTC 1731  
K I M L E D G N L H V T Q G A G R F I [REDACTED] S<sub>1</sub> F 474

TCT CAC TAT GTC TAT AAG CGC ATT AAA GCA AGG AGG AAG ATG GCA GAC CTC CAT CCA GTC CCA AGA GGC ATG 1803  
S D Y V Y K R I K A R R K M A D L H A V P R G M 498

TAT GAT GGA CCA GTG TTT GAC TTG ACC ACC ACC CCC AAG GGG GGC ACC CCA GCT GGC TCT ACT CCG GGC TCT 1875  
Y D G P V F D L T T T P K G G T P A G S T [REDACTED] S<sub>1</sub> 522

CCC ACT CCG CCA AAC CCG CCA GTG AGG AAC CTC CAT CAG TCA GGA TTT AGC CTG TCA GGC ACC CAA GTG GAT 1947  
P T R P N P P V R N L H Q S G F S L S G [REDACTED] 546

GAG GGT GTC CCG TCA GCT AGC AAA CGC ATT GTG GCA CCC CCT GGA GGC CGT TCT AAC ATC ACA TCC CTG AGT 2019  
[REDACTED] G V [REDACTED] S<sub>1</sub> K R I V A P P G G R S N I T S L S 570

caagccctcccaagagggagcagagcaaaaaaactcgaggggg 2076

from purified Ulip, indicating that it indeed encodes this protein. There is no significant homology between the sequences of Ulip and stathmin, suggesting that the cross-reactivity with the anti-stathmin antiserum affected only a restricted epitope and, thus, was fortuitous. The calculated molecular mass of the protein corresponding to the identified sequence is 61,940 Da, and its theoretical pI is 6.3. It is hydrophilic and contains several predicted  $\alpha$  helices and  $\beta$  sheets, but none of them of extensive length.

In agreement with the observation that Ulip is a phosphoprotein *in vivo*, its sequence displays consensus sites for several protein kinases (Fig. 2, shaded boxes) such as PKA (S363, S522, and S553), casein kinase II (T84, S226, and T543), and "proline-directed" kinases such as cdk or mitogen-activated protein kinase (MAPK) (S308, T313, and S472). This is also in agreement with *in vitro* phosphorylation results indicating that the partially purified Ulip protein is a good substrate for casein kinase II, cdc2, and PKA (data not shown).

### Molecular conservation of Ulip and sequence homologies with a D-hydantoinase and Unc-33

A search for protein sequence homology in the GenBank database revealed that the Ulip amino acid sequence is 77% identical to that of the rat neuronal protein designated toad-64 (accession number Z46882) (Minturn et al., 1995). This protein, therefore, likely is a member of the same protein family as Ulip (see also Discussion).

Databank screening also revealed several short, Ulip-related human fetal brain-expressed-sequence tags (ESTs) (Adams et al., 1993), which cover three domains (*EST I*, *EST II*, and *EST III* in Fig. 3) that together represent more than half of the Ulip sequence. There is a high degree of similarity between the corresponding regions of the mouse Ulip and the human EST-derived amino acid sequences (81, 76, and 96% identity within *EST* domains I, II, and III, respectively). Furthermore, the anti-Ulip antiserum recognized a protein with an  $M_r$  of 64 kDa on a Western blot of a human brain extract, as in the mouse (data not shown). Therefore, it is likely that the EST sequences correspond to one or more human proteins of the Ulip protein family (see also Discussion).

GenBank database screening also revealed homologies with sequences from more distant species: a bacterial D-hydantoinase (Jacob et al., 1987), and the product of the *unc-33* gene from the nematode *C. elegans* (Li et al., 1992) (Fig. 4).

The amino acid sequence of the *Pseudomonas putida* D-hydantoinase covers approximately three quarters of the Ulip sequence (residues 14–469), with nearly 40% identity. D-Hydantoinases catalyze the hydrolysis of D-5-mono-substituted hydantoins and D-dihydropyrimidines to yield the corresponding N-carbamyl-D-amino acid (Syldatk et al., 1990). In preliminary experiments, no D-hydantoinase activity was detected with recombinant Ulip produced in bacteria.

The amino acid sequence of the *C. elegans unc-33* gene product covers nearly the entire length of Ulip with 33.5% identity. It has been proposed that Unc-33 plays a role in

axonal guidance and outgrowth in *C. elegans* (Desai et al., 1988; McIntire et al., 1994). Animals bearing the mutated gene are severely uncoordinated, almost paralyzed, and are partially egg-laying-defective. The gene product of *unc-33* exists in three alternatively spliced forms (of 854, 676, and 523 amino acids), which differ only in the length of their N-terminal extension (Li et al., 1992). The sequenced form of Ulip corresponds to the shortest of the three Unc-33 forms.

Of interest, the triple alignment of Ulip with hydantoinase and Unc-33 reveals a highly conserved region of 34 amino acids (Fig. 4, see region b: 380–413 in Ulip) with 59% overall identity among the three sequences (84% between Unc-33 and Ulip, 65% between hydantoinase and Ulip). The similarity between Ulip and Unc-33, a protein of almost the same length, is dispersed throughout the sequence with a lower degree of similarity in the C-terminal quarter (d) of the protein. The shorter D-hydantoinase sequence reveals a slightly higher (40%) similarity with Ulip than Unc-33 (36%) in the N-terminal three quarters (a–c) of the Ulip sequence, but it lacks the C-terminal d region.

### Brain specificity and developmentally regulated expression of Ulip

The striking molecular similarity of Ulip with the Unc-33 protein, which is implicated in the regulation of axonal guidance and outgrowth in *C. elegans*, led us to investigate their potential biological similarities.

Northern blot analysis of total RNA extracts from various tissues (Fig. 5A) revealed a 5.5 kb Ulip mRNA in newborn mice, mostly expressed in the brain and more weakly expressed in the heart and muscle. In the adult, there was no detectable 5.5 kb signal in any of the tissues tested. However, a 2 kb mRNA not observed in the brain was detected in the testis even at high stringency (65°C in 0.1× SSC, 0.1% SDS).

Western blot analysis of the expression of Ulip in various tissues from the neonatal mouse showed that the protein also is expressed almost exclusively in the brain (Fig. 5B). In agreement with the Northern blot results, a much weaker but clear expression was detected in the heart, as was a very weak but still detectable expression in the lung and muscle, possibly attributable, at least in part, to the presence of neuronal cells in the corresponding tissue preparations. In the adult animal, the expression of the Ulip protein could be detected only in the brain, where its expression was at least 20-fold lower than in the neonate, because even a 20-fold longer autoradiographic exposure of the Western blot revealed a weaker signal (Fig. 5B). Also correlating well with the Northern blot results, a very weak band corresponding to the Ulip protein was detected on the Western blot of the adult testis.

In light of the results showing mostly a neonatal brain-specific expression of Ulip, we further examined its brain-expression pattern during the neonatal period of development (Fig. 5C). The protein becomes detectable in the cortex and the striatum at embryonic day 16, reaching a peak at approximately postnatal day 5, when neuronal differentiation and synapse formation are very

**Figure 2.** Complete nucleotide sequence of the cloned cDNA and deduced amino acid sequence of Ulip from mouse brain. The nucleotide sequence is numbered starting from the first nucleotide at the *EcoRI* insertion site. Amino acids are numbered from the ATG start codon after the boxed consensus Kozak sequence. The sequences of peptides I–V determined by protein sequencing are underlined, and the regions corresponding to the two oligonucleotides (*Oli-1* and *Oli-2*) used for the library screening are overlined. Consensus phosphorylation site sequences are indicated in shaded boxes (with the presumed phosphorylation residue in a black box) for PKA, casein kinase II (dashed underline), and "proline-directed" kinases (solid underline).

EST	I-MSYQCKKNIP	RITSRRLIK	GKIVNDQDS	FYADIYMEDG	LIKQIGENLI	VPGGVKTIEA	NGRMVIFOGI	DVNTYLQKPS	QGHTAANDPF	
Ulip	MSYQCKKNIP	RITSRRLIK	GKIVNDQDS	FYADIYMEDG	LIKQIGENLI	VPGGVKTIEA	NGRMVIFOGI	DVNTYLQKPS	QGHTAANDPF	90
toad	MSYQCKKNIP	RITSRRLIK	GKIVNDQDS	FYADIYMEDG	LIKQIGENLI	VPGGVKTIEA	NGRMVIFOGI	DVNTYLQKPS	QGHTAANDPF	
EST	QGTRAAALVGG	TTMIIDHVV	EPGSSLLTSF	EKWHEAADTK	SCCNYSLVVD	ITS-I				143
Ulip	QGTRAAALVGG	TTMIIDHVV	EPGSSLLTSF	EKWHEAADTK	SCCNYSLVVD	ITS-I				143
toad	QGTRAAALVGG	TTMIIDHVV	EPGSSLLTSF	EKWHEAADTK	SCCNYSLVVD	ITS-I				143
Ulip	LYEFTCLGE	LGALIAQVHAE	NGDIIAQSOA	RMLHMGITGP	EGEVLSPREE	LEAEAVFRAI	TVASQTNCPL	YVTKVMSKSA	ADLISQARSK	270
toad	LYEFTCLGE	LGALIAQVHAE	NGDIIAQSOA	RMLHMGITGP	EGEVLSPREE	LEAEAVFRAI	TVASQTNCPL	YVTKVMSKSA	ADLISQARSK	270
Ulip	GNVVGEPIT	ASLGIDGTHY	WSKNWAKAAA	FVTSPPPLSPD	PTTPDYINSL	LASGDLQLSG	SAHCTFSTAQ	KAIGEDMFTA	IPBGTNGVEE	360
toad	GNVVGEPIT	ASLGIDGTHY	WSKNWAKAAA	FVTSPPPLSPD	PTTPDYINSL	LASGDLQLSG	SAHCTFSTAQ	KAIGEDMFTA	IPBGTNGVEE	360
EST	II-V	ATGKMDENQF	VAVTSTNAAK	IFNLYPRKGR	IAVGSADAVV	IWDPOKLKTI	TAKSHKSAVE	YNIFEGMECH	GSPLVVISQG	
Ulip	II-V	ATGKMDENQF	VAVTSTNAAK	IFNLYPRKGR	IAVGSADAVV	IWDPOKLKTI	TAKSHKSAVE	YNIFEGMECH	GSPLVVISQG	450
toad	II-V	ATGKMDENQF	VAVTSTNAAK	IFNLYPRKGR	IAVGSADAVV	IWDPOKLKTI	TAKSHKSAVE	YNIFEGMECH	GSPLVVISQG	
EST	KIVFEDGNIN	VNKGMRFP	RKAFFE-II							522
Ulip	KIVFEDGNIN	VNKGMRFP	RKAFFE-II							522
toad	KIVFEDGNIN	VNKGMRFP	RKAFFE-II							522
EST	ESGTQVDEGV	-RSASKRIVA	PPOGRSNITS	LS-III						
Ulip	ESGTQVDEGV	-RSASKRIVA	PPOGRSNITS	LS-III						570
toad	ESGTQVDEGV	-RSASKRIVA	PPOGRSNITS	LS-III						570

Figure 3. Amino acid alignment of Ulip with human ESTs and rat toad-64. Three human fetal brain EST-derived amino acid sequences obtained by combining overlapping shorter fragments from GenBank (I, combination of ESTs 05414 from Ulip residues 1–60 and EST 04167 from Ulip residues 61–143; II, EST 06020; III, EST 03046; respective GenBank accession numbers are T07524, T06278, T08129, and T05158) and the toad-64 sequence (GenBank accession number Z46882) (toad) were aligned with Ulip. Alignment with the Clustal V software reveals an overall 81% identity between Ulip and the EST fragments (I, 81%; II, 76%; III, 96%) and 76% identity between Ulip and the toad-64 protein. Double identities between sequences are indicated by colons, and triple identities are indicated by stars.

active. It then decreases to very low levels as soon as postnatal day 20.

#### Regulation of Ulip phosphorylation by NGF in PC12 cells

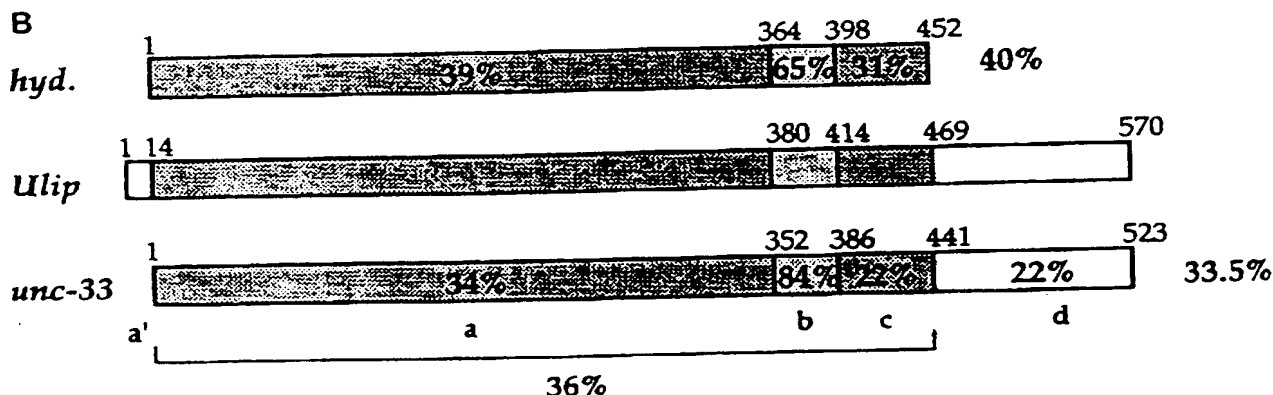
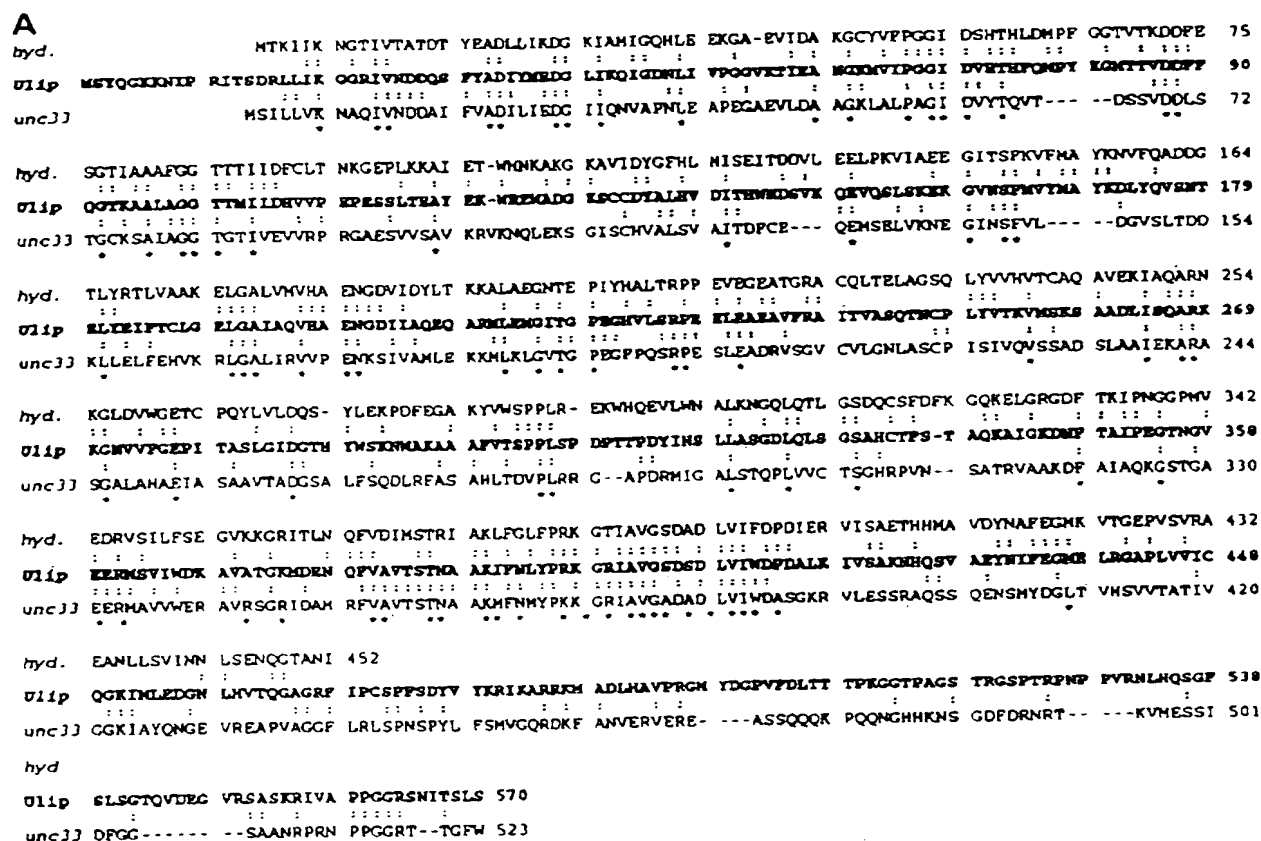
The PC12 cell line often is used as an *in vitro* model for neuron-like differentiation. Indeed, NGF induces the flattening of cells and the outgrowth of neurites as well as the appearance of molecular markers of neurons (Greene and Tischler, 1982).

Two-dimensional Western blots with antisera directed against the mouse protein showed that Ulip is detected easily in rat PC12 cells (Fig. 6B), where it also can be identified clearly on gels by silver-staining (Fig. 6A,B). The rat PC12 protein actually comigrated with the purified mouse brain protein on two-dimensional PAGE gels (data not shown). These results are in good agreement with the apparent evolutionary conservation of the Ulip protein. As in the brain, only the specific anti-Ulip antisera recognized its B isoform. As mentioned above, all antisera also recognized a third likely isoform (C) of the Ulip protein in PC12 cells, with an *M<sub>r</sub>* of ~85 kDa (Fig. 6B), which is expressed at a level comparable with that of the A isoform. This isoform was also detected by silver-staining.

The presumably phosphorylated forms of the C isoform, like the B isoform, have identical isoelectric points to those of the A isoform. It is unlikely that the interaction with the C isoform represents a fortuitous cross-reactivity of three distinct antisera. This isoform, therefore, probably corresponds to another alternatively spliced form of Ulip, including additional exons that might account for its larger size, or to a closely related protein of the same family.

It was of great interest to see whether the phosphorylation pattern of Ulip was changed after induction of the differentiation of these cells toward a neuron-like phenotype. PC12 cells were labeled with [<sup>32</sup>P](PO<sub>4</sub>)<sub>3</sub> *in vivo*, and the radioactive phosphoproteins from control or NGF-induced cells were analyzed by two-dimensional PAGE and autoradiography. As shown in Figure 6C, at least two forms, A3 and A4, were radiolabeled in control cells. These phosphoproteins coincided clearly with the known Ulip spots on silver-stained gels as well as with the immunostained spots on comigrations with the soluble fraction of PC12 cells (data not shown).

After NGF treatment for 30 min, the A4 form became undetectable. This might be attributable either to the phos-



**Figure 4.** Alignment of Ulip with *unc-33* and a D-hydantoinase. **A**, Triple alignment of the amino acid sequences of Ulip with those of the *C. elegans* *unc-33* gene product and of the D-hydantoinase (*hyd.*) from *P. putida* is shown. Double identities between sequences are indicated by *colons*, and triple identities are indicated by *stars*. **B**, Regions are delimited according to their degree of similarity (*percentages* refer to identities with the sequence of Ulip within each region or at right within their whole length). Note the well preserved *b* region, presenting 59% overall identity among the three proteins.

phorylation of A4 to a highly phosphorylated form or to the dephosphorylation of A4. Based on their migration pattern, the three spots indicated by *stars* above A3 and A4 on Figure 6C most likely correspond to forms B4a, B5, and B6. They were heavily phosphorylated under control conditions, whereas the addition of NGF induced a decrease in the intensity of the two more acidic spots.

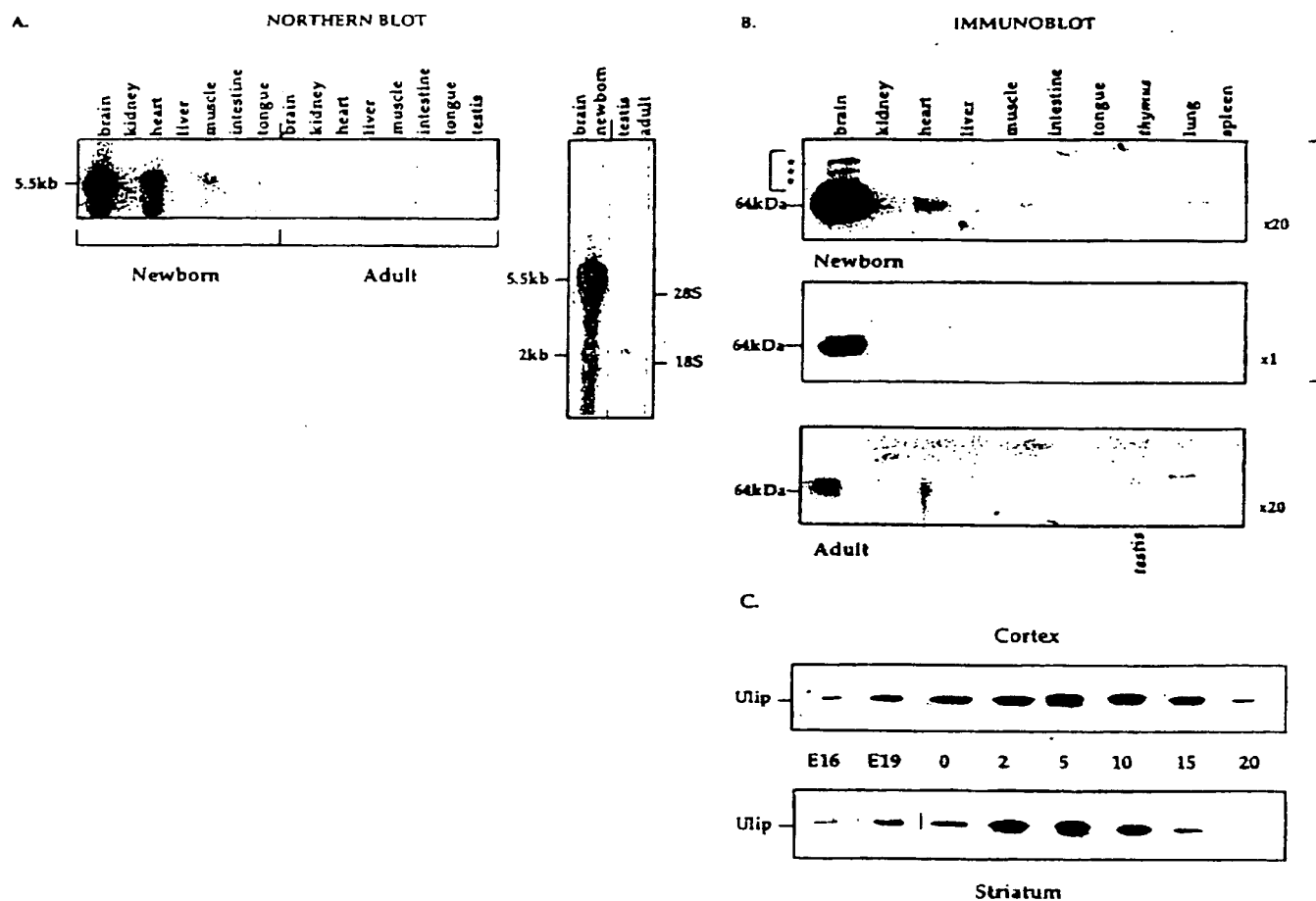
It appears, therefore, that the phosphorylation of Ulip forms in PC12 cells is modified in response to NGF, possibly in

relation to the control of neuronal differentiation and neurite outgrowth.

#### Intracellular distribution of Ulip in PC12 cells and in primary cultured neurons

To attain deeper insight into the possible biological role of Ulip, its intracellular distribution in PC12 cells and in cultured rat striatal neurons was examined by immunofluorescence with the anti-recombinant Ulip antiserum (Fig. 7). When the same cultured neurons were labeled





**Figure 5.** Tissue specificity and developmental regulation of the expression of Ulip in the mouse. **A**, Northern blot. Five micrograms of total RNA of the indicated tissues from newborn or adult mouse were separated on a 1% agarose gel containing formaldehyde, transferred to nylon membranes, and hybridized with the  $^{32}$ P-labeled *EcoRV*-*XhoI* fragment of the Ulip cDNA (see Materials and Methods). The integrity and the quantitative equivalence of the RNAs were checked before hybridization by staining with methylene blue (data not shown). The right panel displays the entire length of the first and the last lanes of the left panel (newborn brain and adult testis), showing the weak 2 kb signal observed only in adult testis. **B**, **C**, Immunoblots. **B**, Sixty micrograms of protein homogenates from the indicated tissues were separated by one-dimensional PAGE on a 10% polyacrylamide gel, transferred onto nitrocellulose membranes, and probed with the anti-recombinant Ulip antiserum (1:50,000) and detected with  $^{125}$ I-labeled protein A. As indicated, all of the tissues examined in the newborn and adult animals were the same except for the thymus in the newborn and the testis in the adult mouse. The immunoblots were exposed either without ( $\times 1$ ) or with ( $\times 20$ ) a Kodak Quanta III screen for the same duration, the latter yielding an  $\sim 20$ -fold enhanced signal. Stars refer to the minor, higher  $M_r$  bands visible in the newborn brain sample, most likely corresponding to the B and C Ulip isoforms (see Figs. 1, 6). **C**, Fifty micrograms of protein from mouse brain cortex or striatum homogenates at the indicated stages of development were separated by one-dimensional PAGE, transferred onto nitrocellulose membranes, and revealed with cross-reactive anti-stathmin antibody (1:10,000) and  $^{125}$ I-labeled protein A, showing a peak of Ulip expression at  $\sim 5$  d postnatal.

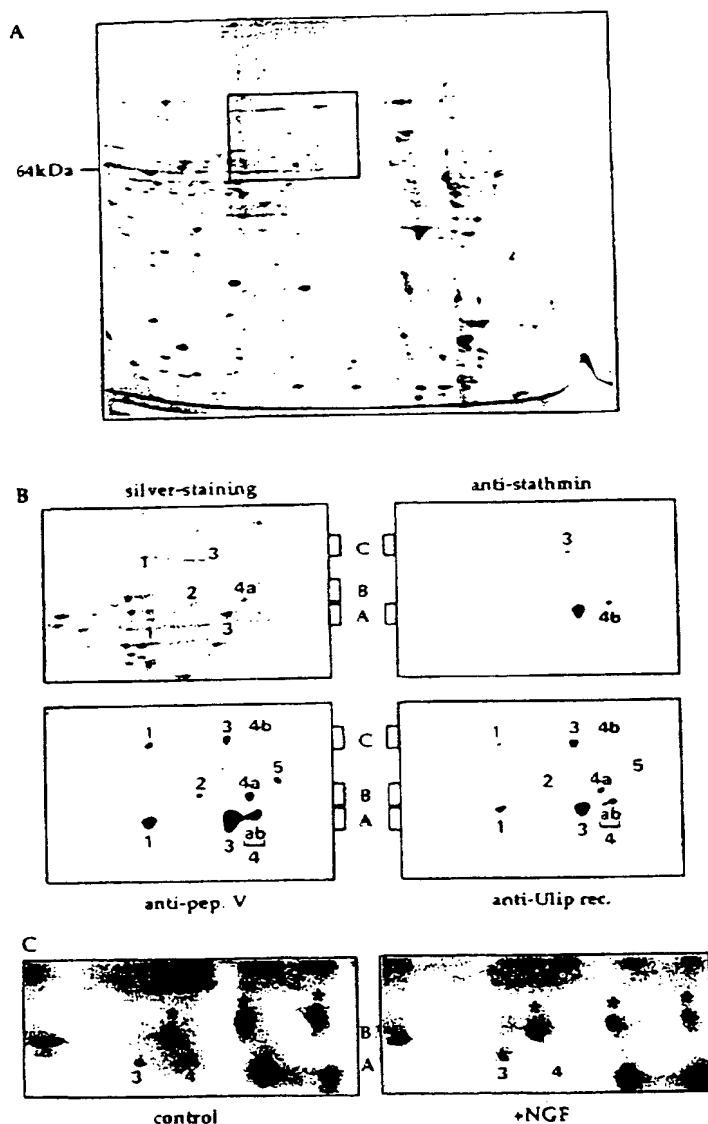
either with immune serum preincubated with the recombinant Ulip protein or with preimmune serum at the same dilution, the fluorescence was reduced strongly, indicating that the labeling with the anti-Ulip serum was highly specific.

In PC12 cells, the protein was cytoplasmic and was absent from the nuclei. After induction of differentiation with NGF, Ulip was also detectable in all of the neurites, where the antiserum clearly labeled varicosities (arrowheads; the lower intensity of neurite labeling in Fig. 7B is attributable to the focus on the cell body and nucleus). In agreement with results from immunoblot experiments (data not shown), no difference was observed in the expression level of Ulip between NGF-treated and nontreated cells. No clear colocalization of the Ulip protein with cytoskeletal elements could be observed by double-staining PC12 cells with antibodies recognizing cytoskeletal proteins such as tubulin, actin, and the  $M_r$  68 kDa neurofilament isoform (data not shown).

Primary neurons from E20 rat striatum were examined after 8 d in culture (Fig. 7). Like PC12 cells, all of the neurons displayed immunoreactivity in the cytoplasm, whereas very little if any signal could be detected in the nuclei. The neurites and axons were labeled along their entire length, with a strong staining at the varicosities (arrowheads) and at the growth cones (arrows).

#### Expression of Ulip at the neuromuscular junction

The fact that Ulip was associated with neuritic processes grown in culture prompted us to examine its presence at peripheral motor nerve endings at the neuromuscular junction. Double-labeling experiments were performed using the anti-Ulip antiserum and the rhodamine-conjugated  $\alpha$ -bungarotoxin, which binds to acetylcholine receptors (Fig. 8). Synaptic sites, which were identified by their  $\alpha$ -bungarotoxin labeling on cross-sections of adult gastrocnemius muscle, displayed a moderate but clearly positive Ulip



**Figure 6.** Ulip expression and phosphorylation in PC12 cells. Proteins from the soluble fraction of PC12 cells were separated by two-dimensional PAGE. The entire silver-stained two-dimensional gel is shown with the area of Ulip boxed (*A*). In *B*, the same area enlarged is displayed together with the corresponding areas of Western blots probed with sera directed against stathmin, peptide V, or recombinant Ulip. Numbers refer to the various forms of each of the A (64 kDa), B (70 kDa), and C (85 kDa) isoforms of Ulip, as defined in Figure 1. Note that the A and C isoforms are recognized by all the three antisera, whereas the B isoform is not recognized by the anti-stathmin antiserum. For this experiment, the anti-peptide V antibody was used after affinity purification against peptide V. *C*, *In vivo* phosphorylation in PC12 cells. PC12 cells were incubated for 4 hr with  $^{32}$ P-labeled  $\alpha$ -phosphate without (control) or with (+NGF) the addition of NGF (200 ng/ml) for the final 30 min. Proteins were separated by two-dimensional PAGE and detected by autoradiography. Numbers refer to the corresponding phosphorylated forms of the A isoform of Ulip, whereas the asterisks indicate most likely increasingly phosphorylated B forms.

staining. Triple labeling with anti-Ulip, anti-neurofilament antibody, and  $\alpha$ -bungarotoxin showed that Ulip immunoreactivity was associated with intramuscular nerve branches located near the neuromuscular junction and was recognized by the positive staining with the anti-neurofilament antibody (data not shown).

To confirm further the location of Ulip at the neuromuscular junction, we performed whole-mount preparations of teased sternocleidomastoid muscle fibers (Fig. 8). Ulip immunoreactivity examined by confocal fluorescence microscopy appeared at two different levels at motor nerve endings: as a staining pattern that closely matched the  $\alpha$ -bungarotoxin labeling, and as a positive immunoreaction delineating the preterminal region associated with the neuromuscular junction.

## DISCUSSION

The present characterization of the developmentally regulated neuronal Ulip phosphoprotein led us to identify both molecular and biological similarities with the product of the *C. elegans* gene *unc-33*. Ulip thus is likely to be its functional mammalian homolog, potentially related to the molecular processes underlying axonal guidance and neuritic elongation that take place during development and regeneration within the nervous system.

### Biological and molecular properties of Ulip

The expression of Ulip appeared preferentially in the nervous system, where it is highly dependent on development; these findings support a role of Ulip related to the molecular processes allowing and/or controlling specific stages of neuronal development.

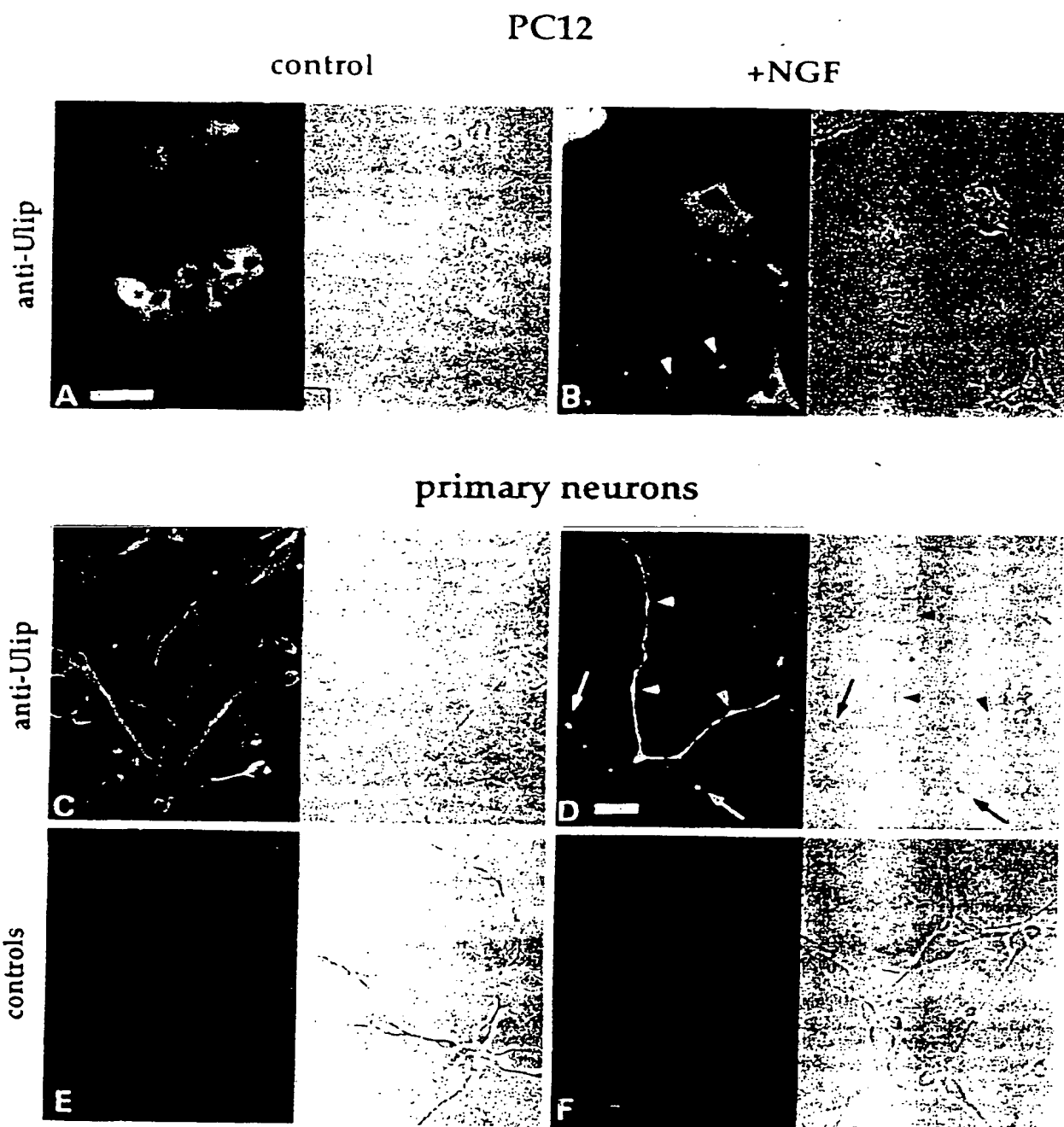
In PC12 cells and primary neurons in culture, Ulip could be detected in the cell body, but not in the nucleus, and in all of the neuritic processes, showing a marked labeling of varicosities and growth cones. These observations clearly demonstrate the neuronal expression of Ulip and its likely involvement in neuronal growth and/or differentiation. Ulip was detected in peripheral nerve endings at the adult neuromuscular junction, where it was coincident with the nicotinic acetylcholine receptor labeling, but Ulip was associated also with presynaptic regions. This latter observation reflects the presence of Ulip in neuronal processes and, possibly, in associated terminal Schwann cells.

In the mouse brain, at least two Ulip isoforms, A and B, are present, with respective  $M_r$  values of 64 and 70 kDa, whereas a third, additional  $M_r$  85 kDa C isoform is expressed in rat PC12 cells. In the mouse brain, a single 5.5 kb mRNA was detected, which likely codes for the most abundant A isoform. The various forms of Ulip might be products of the same gene and translated from alternatively spliced mRNAs, as was shown also for *Unc-33* (Li et al., 1992). Another possibility is that the diverse forms recognized by the various antisera correspond to closely related proteins of a common, conserved family, as suggested by the comparison of all of the related sequences available so far (see below). Interestingly, an additional mRNA of ~2.0 kb was found in the testis, where traces of the protein also were detected.

Ulip is a phosphoprotein, because it is sensitive to alkaline phosphatase and incorporates radioactive phosphate, which is in good agreement with the presence of consensus phosphorylation sites for several types of protein kinases within its amino acid sequence. *In vivo*, the phosphorylation state of Ulip in PC12 cells was modified in response to the neuronal growth, differentiation, and survival factor NGF. This is a clear indication of a possible regulation of the activity of this protein, and of its potential implication in intracellular cascades involved in the regulation of neuronal differentiation and maturation.

### Molecular and functional similarities

The databank search with the Ulip sequence revealed similarities with mammalian sequences, toad-64, several brain ESTs

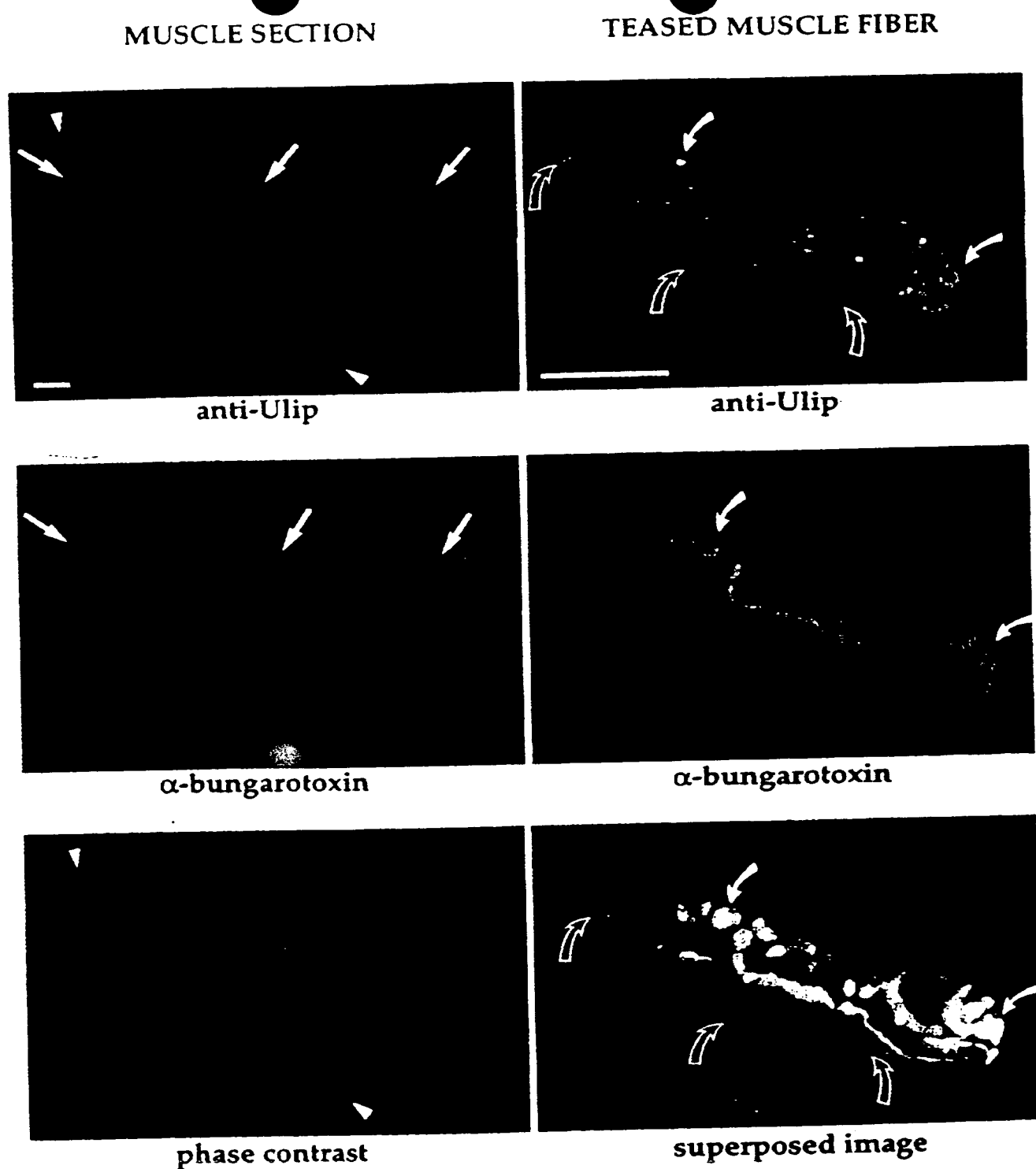


**Figure 7.** Immunofluorescence labeling of Ulip in PC12 cells and rat striatal primary neurons. Control (A) and NGF-induced (B) PC12 cells were fixed with methanol, and E20 neurons from rat striatum (C–F) were fixed after 8 d in culture with 2% *p*-formaldehyde. Cells were labeled with the anti-recombinant Ulip antiserum (1:500; A–D), with anti-Ulip antiserum preincubated with 10  $\mu$ g/ $\mu$ l recombinant Ulip protein (E), or with preimmune rabbit serum at the same dilution as in A–D. Primary antibodies were revealed with rhodamine-conjugated anti-rabbit secondary antibodies. Note neurites with strongly immunoreactive varicosities (arrowheads) and growth cones (arrows). In B the focus is mainly on the cell body, resulting in lower apparent fluorescence intensity in neurites; in D, only the axonal extension of a neuron can be seen, the cell body being outside the field. Scale bars, 25  $\mu$ m; magnification in B, C, E, and F as in A.

and, more provocatively, a *P. putida* bacterial hydantoinase and the *C. elegans unc-33* gene product. After submission of this paper, two additional Ulip-related sequences were reported, crmp-62 in chick (Goshima et al., 1995) (accession number U17277) and munc in mouse (S. Tonitsch, unpublished data; accession number X87242).

Ulip exhibits puzzling sequence similarities with several bacterial hydantoinases (Yang et al., 1993), the most striking of them

with an enzyme from *P. putida* (Jacob et al., 1987), the sequence of which covers the N-terminal three quarters of Ulip. The only mammalian hydantoinase-like protein that displays a certain similarity with Ulip is a hamster dihydro-orotase (Simmer et al., 1990; Williams et al., 1990), which has a high degree of similarity (60% identity) with Ulip only in a 37-amino-acid stretch (Ulip residues 67–103). This region is well preserved in all dihydro-orotases known so far, but the probable critical amino acids for  $Zn^{2+}$



**Figure 8.** Ulip expression at the neuromuscular junction. Transverse sections of adult mouse gastrocnemius muscle (*left*) or whole-mount-teased fibers from sternocleidomastoid muscle (*right*) were double-stained with the anti-recombinant Ulip antibody (revealed with a fluorescein-conjugated secondary antibody) and rhodamine-conjugated  $\alpha$ -bungarotoxin. On the muscle sections examined by conventional fluorescence microscopy, immunoreactivity to antibody was present at the synaptic sites (arrows) identified by  $\alpha$ -bungarotoxin binding as well as on interstitial nerve fibers (arrowheads). Confocal microscopic analysis of teased muscle fibers revealed that Ulip immunoreactivity entirely recovered the distribution of acetylcholine receptors (filled curved arrows), thus appearing yellow on the superposed image. It was present additionally in scarce extrasynaptic regions revealed by the green staining on the superposed image (open curved arrows). Scale bars, 25  $\mu$ m.

binding (Williams et al., 1990) are not fully conserved in Ulip. Because the rest of the dihydro-orotase sequence shows almost no homology with Ulip, it is not likely that Ulip corresponds to this enzyme. The fact that no hydantoinase activity could be detected in preliminary experiments with recombinant Ulip also does not support a direct functional homology with this class of enzymes. However, the observed similarities might correspond to the conservation of a functional, enzymatic, or regulatory domain related to the involvement of Ulip in a step of neuron-specific metabolism important for neuritic outgrowth and axonal guidance during neuronal differentiation.

In view of the neuronal and developmental expression of Ulip, its sequence similarity with Unc-33 seems to be more directly relevant at the functional level. Three forms of Unc-33 are expressed in *C. elegans*, which are the products of differentially spliced mRNAs with  $M_r$  values of 55.5, 72.1, and 90.8 kDa, differing only in the length of their N-terminal extensions (Li et al., 1992) and corresponding to the expression of three mRNAs of 2.8, 3.3, and 3.8 kb, respectively. The amino acid sequence of the shortest Unc-33 form covers the entire sequence of the presumably shortest, A form of Ulip, with an overall identity of 33.5%. Although there are clearly several genes for Ulip-related proteins, it is possible that some of the Ulip forms with molecular sizes in the mouse or rat comparable with those found for *unc-33* isoforms in *C. elegans* are derived also by alternative splicing of a given gene.

In *unc-33* *C. elegans* mutants, nearly all neurons inspected, including sensory, motor, and interneurons, show neuritic outgrowth defects (Hedgecock et al., 1985; Desai et al., 1988); some neuritic processes follow abnormal pathways and have swollen endings and prematurely terminated axons, which altogether underline the developmental importance of the Unc-33 protein. The developmental regulation of Ulip expression, mostly at a stage at which neuronal differentiation and maturation take place, indicates that its molecular similarity is indicative of a functional similarity with Unc-33 and, therefore, that it also might play a role in the control of neurite elongation and/or axonal guidance. The relatively low-level presence of Ulip in the adult brain might reflect its presence in areas where neurogenesis remains active or its involvement in maintaining neuronal stability, for example, at the junctional level. This latter possibility is supported by the presence of Ulip in adult nerve endings, as at the neuromuscular junction.

Interestingly, the two other Unc-33-related sequences reported in the literature, toad-64 in the rat (Minturn et al., 1995) and *crmp-62* in chick (Goshima et al., 1995), are expressed like Ulip mostly during development in the nervous system but, in contrast with Ulip, they were not detected in any other tissue even at low levels. Comparison of their amino acid sequences reveals that toad-64, *crmp-62*, and *munc*, a mouse sequence that recently became available in the sequence databanks, display ~97% amino acid identities, whereas Ulip is only ~76% identical to any of the other three sequences. This observation reveals, as suspected on the basis of the protein expression profiles discussed above, that Ulip is a member of a protein family: it should be designated, therefore, as Ulip1, the other group of Unc-33-related sequences then being Ulip2—highly conserved between birds (*crmp-62*) and mammals (toad-64 and *munc*). Several human ESTs are at least 96% identical either with Ulip1 or with the various known Ulip2 sequences, further demonstrating the high evolutionary conservation of each of these two members of the Ulip protein family. Additional human EST sequences display 72–76% identity with

either Ulip1 or Ulip2: they belong to the partial sequence designated *Hcrmp-1* by Strittmatter and coworkers (Goshima et al., 1995), which covers the Ulip1 and -2 sequences from their residue 59 to their C-terminal extremity. The corresponding protein could be designated, therefore, as Ulip3, to indicate its clear inclusion in the same protein family. The recognition of these three members of the Ulip family allows us to understand further the correspondence of the three EST domains defined in Results and shown in Figure 3: EST I (1–60) corresponds to Ulip2; EST I (61–143) and EST II correspond to Ulip 3; and EST III corresponds to Ulip1. Finally, some ESTs display lower degrees of similarity (50–60% identity) with the above-described Ulip sequences, caused by sequencing errors or by reflecting the existence of more members of the Ulip family. This overall analysis of available sequences also might explain some of our results concerning the expression of Ulip mRNA or protein, particularly in non-neuronal tissues and potentially in non-neuronal cells within the nervous system. It is likely that, although various members of the Ulip family are expressed most highly in neuronal cells, they also have distinct expression patterns, with respect both to the cell type considered and to differentiation stages during development.

We characterized Ulip as highly regulated during developmental stages in the mouse brain corresponding to active neurogenesis and synapse formation; its regulated phosphorylation suggests further its potential implication in the intracellular cascades responsible for the relay and integration of signals controlling neuronal differentiation. The present molecular and biological characterization of Ulip (Ulip1) strongly indicates that it is a structural and functional mammalian homolog of Unc-33. It appears, therefore, to be a good candidate for participating in neuronal differentiation and maturation, presumably through the control of neuritic elongation and/or of axonal guidance during the development of the nervous system. Our data suggest further that Ulip plays a role during development in other tissues and cell types, and in the adult, where it might participate, for example, in the normal or post-traumatic replacement and regeneration of neurons; Ulip also is involved potentially in the promotion or maintenance of the stability of neuronal connections.

Toad-64 was recognized to be upregulated very early after division of neuronal cells and was considered to be an early marker of cells committed to a neuronal phenotype (Minturn et al., 1995). *Crmp-62* was suggested to be involved in the intracellular cascade initiated in response to collapsin (Goshima et al., 1995). Most of the ESTs corresponding to Ulip3 were identified from a human embryonic brain library (Adams et al., 1993), suggesting an expression pattern and a developmental regulation at least partially similar to those of Ulip1 (Ulip) and Ulip2 (toad-64 and *crmp-62*). However, the actual function(s) of each of the vertebrate proteins of the Ulip family is likely to be of broader significance than those originally proposed for toad-64 and *crmp-62*. Because all Ulip-related vertebrate proteins display functional and molecular similarities with the *C. elegans unc-33* gene product (32–35% sequence identity), we believe that their collective designation as "Ulip" proteins (1, 2, 3, ...) is the most appropriate because it refers to their proven resemblance to Unc-33. Their identification will allow the further characterization of their common and differential biological functions.

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om masking studies<sup>18</sup>. Our results further suggest that the ering stage is directionally selective. Early experiments<sup>19,20</sup> aptation suggested that radial and translational motion be analysed by different mechanisms<sup>19,20</sup>, although the ative predictions of the size of the receptive fields for mechanisms (less than 1.5°) agrees neither with the results ed here nor with the physiology<sup>4,7</sup>; perhaps first-stage nisms may have mediated the adaptation to some extent. te these differences, however, the adaptation studies also sted that radial motion may be a second-stage analysis. a common contrast-dependent first stage<sup>21</sup>. Modern electrophysiological studies have shown that motion cessed at several different cortical levels, including primary l cortex (V1), middle temporal (MT) and medial superior al (MST) cortex. Neurons in V1 are selective to transla- l motion, and have relatively small receptive fields. In MT, tive fields are larger, but the neurons remain selective to lational motion<sup>22</sup>. In the dorsal segment of MST (MSTd), ave very large receptive fields, and many are selective to l or circular motion<sup>4</sup>. However, selectivity is not restricted ese 'cardinal directions' of optic flow, as there also exist ns selective to combinations of radial and circular motion al motion) and to combinations of spiral and translational on<sup>7</sup>. The experiments reported here do not address the of whether selectivity is restricted to the cardinal directions. lo show that there must exist neurons that respond to radial ricular motion trajectories. Other properties of MSTd neu- , such as their insensitivity to density, texture and relative h of the texture elements<sup>4,5</sup>, and particularly to the position

of the centre of the radial, circular or spiral motion<sup>6,7</sup> (also supported by psychophysical evidence (R. J. Snowden and A. B. Milne, manuscript submitted)) make these neurons well suited for optic-flow field analysis, and for guiding the heading of ego motion<sup>23,24</sup>.

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## Collapsin-induced growth cone collapse mediated by an intracellular protein related to UNC-33

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COLLAPSPIN<sup>1</sup>, a member of the newly recognized semaphorin family<sup>2–4</sup>, contributes to axonal pathfinding during neural development by inhibiting growth cone extension<sup>1–3</sup>. The mechanism of collapsin action is poorly understood. Here we use a *Xenopus laevis* oocyte expression system to identify molecules involved in collapsin signalling, because several experiments have raised the possibility that heterotrimeric GTP-binding proteins might participate in these events<sup>5–9</sup>. A collapsin response mediator protein of relative molecular mass (*M<sub>r</sub>*) 62K (CRMP-62) required for collapsin-induced inward currents in *X. laevis* oocytes is isolated. CRMP-62 shares homology with UNC-33, a nematode neuronal protein required for appropriately directed axonal extension<sup>10–12</sup>. CRMP-62 is localized exclusively in the developing chick nervous system. Introduction of anti-CRMP-62 antibodies into dorsal root ganglion neurons blocks collapsin-induced growth cone collapse. CRMP-62 appears to be an intracellular component of a signalling cascade initiated by an unidentified transmembrane collapsin-binding protein.

To characterize components of the collapsin response pathway, we injected RNA synthesized from a chick E7 dorsal root ganglion (DRG) complementary DNA library into oocytes, and then assessed the current response of voltage-clamped oocytes to bath application of collapsin. With collapsin-containing chick E10 brain membrane extracts (BME) (refs 6, 13) as ligand, a cDNA clone capable of rendering oocytes responsive was identified (not shown). This clone consists of 300 bp of 5' untranslated sequence followed by 890 bp of open reading frame (ORF) and no stop codon. We obtained a 3' extension of this clone by polymerase chain reaction and ligated the two fragments together to create CRMP-62. To determine if collapsin is the active component of BME in this assay, we expressed collapsin in COS cells and insect cells. Purified recombinant collapsin from baculovirus-infected cells has a specific activity of 10<sup>6</sup> U mg<sup>-1</sup> protein and produces pertussis toxin (PTX)-sensitive DRG growth cone collapse (Fig. 1a). The inward current in CRMP-62 RNA-injected oocytes initiated by BME (not shown), collapsin-expressing COS cell medium (Fig. 1b), and pure collapsin (not shown) are indistinguishable.

The CRMP-62 open reading frame encodes a protein of *M<sub>r</sub>* 62K that has no signal peptide or membrane-spanning domain (Fig. 1c). The structure of CRMP-62 suggests that it is an intracellular protein, and that it may be required for coupling a transmembrane collapsin-binding receptor to a signalling cascade. Therefore naive oocytes must have low levels of an endogenous collapsin receptor that generates a current response only in the presence of CRMP-62. Crude DRG messenger RNA injection yields a larger response than can be accounted for by CRMP-62 alone, suggesting that DRG mRNA may encode both a collapsin-binding receptor and CRMP-62.

CRMP-62 is a novel sequence, sharing homology with three groups of sequences. The highest level of similarity is to several human fetal brain expressed sequence tags<sup>14</sup>. There are two human cDNAs, which we term *hCRMP-1* and *hCRMP-2*, sharing 84% and 98% predicted amino-acid identity with CRMP-62 (Fig. 1, and not shown). CRMP-62 is also related to *unc-33* (ref.

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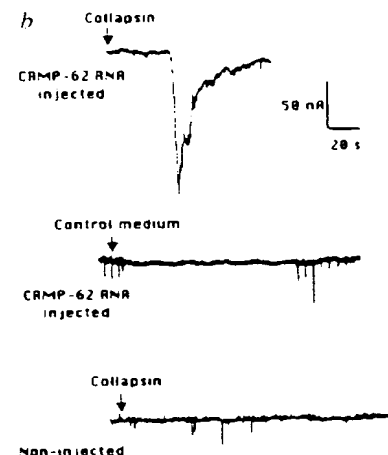
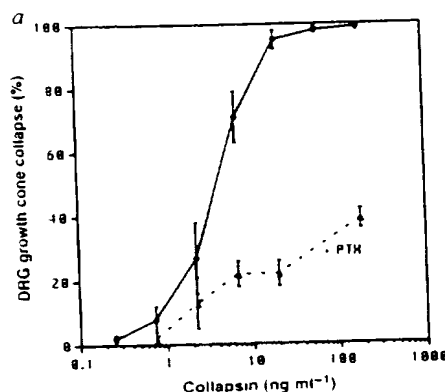
10), with 38% identity and 61% similarity (Fig. 1c). Without *unc-33*, nematode movements are uncoordinated and axon outgrowth is aberrant<sup>11,12</sup>, reminiscent of the disordered outgrowth of insect neurons with perturbations of semaphorin expression<sup>2,5</sup>. Although *CRMP-62* has similarity to bacterial  $\alpha$ -hydantoinase proteins<sup>15,16</sup>, purified recombinant *CRMP-62* has no detectable hydantoinase activity ( $<5$  nmol hydantoin hydrolysed per min at 37 °C per mg protein).

We sought to determine whether *CRMP-62* reconstitutes a collapsin-G protein-phospholipase C-InsP<sub>3</sub>-Ca<sup>2+</sup>-chlor-

ide channel cascade<sup>17</sup>. The reversal potential for BME-induced currents is -21 to -25 mV, identical to that for chloride (not shown). BME responses are abolished by prior injection of EGTA into oocytes (not shown), as expected for a Ca<sup>2+</sup>-mediated event. Furthermore, PTX decreases collapsin-induced currents (Fig. 2a). We also considered whether *CRMP-62* RNA injection might enhance G protein-coupled transduction generally, downstream of G protein activation. However, currents induced by lysophosphatidic acid (LPA) (Fig. 2b) and 5HT (not shown) are identical in non-injected and *CRMP-62* RNA-

FIG. 1 a, Collapse of DRG growth cones by purified collapsin is inhibited by PTX. Explants of E7 DRG were pretreated for 2 h with 500 ng ml<sup>-1</sup> PTX (broken line) or no additions (solid line). Different concentrations of purified collapsin were added and the fraction of collapsed growth cones was determined after 30 min incubation. For concentrations of collapsin greater than 10 ng ml<sup>-1</sup>, PTX reduced growth cone collapse significantly ( $P \leq 0.01$ , Student's two-tailed t test). The data are the mean  $\pm$  s.e.m. for 6 determinations. b, Collapsin induces an inward current in oocytes injected with *CRMP-62* RNA. Oocytes injected with *CRMP-62* RNA or not injected were voltage clamped after incubation for 2 days. Medium from COS cells transfected with pcDNA-collapsin was added at the time indicated by the arrow. An inward current is apparent in *CRMP-62* RNA-injected oocytes but not in control oocytes. Medium from COS cells transfected with pcDNA-1 did not alter membrane conductance. Non-injected control oocytes show no response to purified recombinant collapsin, even at concentrations as high as 200 U ml<sup>-1</sup> ( $>3$  nA in 45 oocytes). Oocyte current responses and chick DRG growth cone collapse exhibit a similar dose response for recombinant collapsin (not shown). The amplitude of current response in *CRMP-62* RNA-injected oocytes is similar to that in oocytes injected with an equal amount of total DRG poly(A)<sup>+</sup> RNA (not shown). c, An alignment comparing the predicted amino-acid sequences of *CRMP-62* and *UNC-33*. Identical residues are shown in bold. The *UNC-33* sequence is from GenBank, accession number Q01630 (ref. 10).

METHODS. Chick E7 DRG growth cone collapse assays were conducted as described<sup>6</sup>. Chick E10 BME was prepared as described<sup>6</sup>, and dialysed extensively against the oocyte perfusion buffer. Collapsin was expressed in COS cells essentially as described<sup>1</sup>, except that transfected cells were cultured in serum-free medium and a pcDNA-1-based vector was used. One unit of collapsing activity is defined as the amount required in a 1-ml culture to collapse 50% of DRG growth cones<sup>1</sup>. The specific activity of this COS cell medium was approximately 500 U mg<sup>-1</sup> protein. A recombinant baculovirus expressing collapsin with an additional six carboxy-terminal His residues was propagated in High Five *Spodoptera frugiperda* cells. After 68 h of infection, cultures were centrifuged at 1,000g for 10 min, and then the low-speed supernatant was centrifuged at 150,000g for 1 h. Nearly all growth cone collapsing activity was present in the high-speed pellet, but after resuspension in PBS plus 1 M NaCl and a second high-speed centrifugation, activity was released into the supernatant. The extracted collapsin was diluted to 150 mM NaCl and chromatographed over S-Sepharose as described for brain extracts<sup>1</sup>. Those fractions containing collapse activity were more than 95% pure by SDS-PAGE (not shown). From a 1-litre culture, 400 to 800  $\mu$ g protein was obtained. *X. laevis* oocytes were prepared, injected and voltage clamped as described<sup>18</sup>. Then, 2-4 days after RNA injection, collapsin (1-10 U ml<sup>-1</sup>) was applied by mixing with the bath medium. Capped RNA was transcribed from a chick E7 DRG pcDNA-1-based cDNA library with T7 RNA polymerase and m7G(5')ppp(5')G. With BME as a ligand, 20 pools of 2,000 recombinants were screened to isolate clone 71. Because clone 71 was truncated at the 3' end, we used chick E7 DRG-derived cDNA as a template for PCR with oligo(dT) and a sense oligonucleotide 150 bp from the 3' end of clone 71. The major reaction product of 1.4 kb contained 150 bp of clone 71, then a NotI site. 810 bp of open reading frame, a stop codon, polyadenylation signal and a polyadenylation tract. Clone 71 was truncated because NotI was used during library construction. To construct pCRMP-62, clone 71 and the PCR-derived 3' fragment were ligated together at this NotI site. Databank searches revealed 8 human ESTs with homology to *CRMP-62*



<i>CRMP-62</i>	MSYQGGKNIP	RITSRLIK	GGKIVHDDQS	YADIYHDD	LKQIGENL	49
<i>UNC-33</i>	MSILAVK	NAQIVHDAI	YADILHDD	LQNVAPME		
<i>CRMP-62</i>	VPGQVKTEA	HGRKVIHGGI	DVHTFQHP	QOHTSADFF	QOTKALAGG	49
<i>UNC-33</i>	APGAEVLDG	AGKALPAGI	DVYTLQVTO	LLSEVDDLS	TCKSLAGG	
<i>CRMP-62</i>	YTHIDHVP	EPGTELLTAF	DQWRWADSK	SCDDYSLHD	ITETHKGVQE	149
<i>UNC-33</i>	TGTIVVYRF	RGAEVSVSAV	KRVKLNQLEK	SGISCHVAL	SVAITDFEQ	
<i>CRMP-62</i>	IKKALVHKG	VNSPLVYMAF	KRFQSLDSQ	LYEVLSVIR	QATAYHVAE	199
<i>UNC-33</i>	IKSELVKNK	INSPVL...	DOVSLTDOK	LLSEFHVKR	LGALLRVVPE	
<i>CRMP-62</i>	MGDIHAEQ	QRILELQITQ	PRGHVLSUPE	SVYEAHVNA	ITIANQTHCP	249
<i>UNC-33</i>	IKKSTVAHL	KKMLKLVTO	PRGFPOSUPE	SLKADPVSGV	CLVGLNLSAP	
<i>CRMP-62</i>	LYETKWHKS	AAEVIAQAK	KOTVYCEPI	TASLGTDGSH	YKKNWAKAA	299
<i>UNC-33</i>	ISTVQVSSD	SLAAEKARA	SGALAHAECA	SAVTAADGSA	LFQDLRFAS	
<i>CRMP-62</i>	AEVTSPLSP	DPPTDFENS	LLSCDQLOV	GSARCTFMTA	QKAVGIDNFT	349
<i>UNC-33</i>	AHLTDVPLR	G...ADPNIG	ALSTQPLVVC	TSGRPVMSA	TRVAARDPA	
<i>CRMP-62</i>	LIPGDTNTE	ERMSIMDKA	VYTKHDENO	FVAVTSTHAA	KIPNLYPRKQ	399
<i>UNC-33</i>	IAQKSTGAS	ERMAVVMERA	VRSGRIDAMR	FVAVTSTHAA	KIPNLYPRKQ	
<i>CRMP-62</i>	RIAVGSDAL	VTDVDSVKT	ISAKTHNISL	EYNIFEGMEC	RGSPVIVISQ	449
<i>UNC-33</i>	RIAVGADAL	VTDVDSGRV	LESSRAQSSQ	ENSMYDQLV	HSVLTATVQ	
<i>CRMP-62</i>	GKIVLEDDNL	HVTEGSRYSI	PRKFPDFVY	KRIKARSRLA	ELRGVPIGLY	499
<i>UNC-33</i>	GKIAVQNGEV	REAPVAGGFL	RLSPKSPYLE	SNVQGRKFA	NYRVEREAS	
<i>CRMP-62</i>	DGPVCEVST	PKTVPASSA	KTSAPAKQAP	PVRNLHOSGF	SLSGAQIDDN	549
<i>UNC-33</i>	QQQKPPQNG	HKNSGDFR	NRTKTMESI	DFQGSANRP	ENPPQGRTO	
<i>CRMP-62</i>	IPRRTTORIV	APPGGRANIT	SLG			
<i>UNC-33</i>	FW					

(GenBank T06278, T08139, T08129, T09404, T06728, T07524, T05711 and M85617)<sup>14</sup>. We obtained plasmid clones for three of these from ATCC, and determined their nucleotide sequence. GenBank T06278 (ATCC 82946), which contains a 1.9-kb insert with a 1.6-kb open reading frame, which we termed *hCRMP-1*. Five of the ESTs (T06278, T08139, T08129, T09404 and T06728) are identical to portions of this *hCRMP-1* sequence. The overlapped combination of open reading frames from ATCC 84159 and ATCC 82402 is the sequence we term *hCRMP-2*. Nucleotide sequences of double-stranded DNA were determined by the dideoxy chain termination method. The GenBank accession numbers for *CRMP-62*, *hCRMP-1* and *hCRMP-2* are U17277, U17278 and U17279, respectively.



injected oocytes. CRMP-62 protein expressed 2 days after RNA injection might participate directly in collapsin signalling, or might chronically alter the transcription of other proteins. To distinguish these hypotheses, we purified recombinant CRMP-62 (Fig. 2c) and then injected 10–30 ng CRMP-62 protein into each oocyte to yield a concentration of CRMP-62 at or below physiologic levels (see below). Then, 5 minutes after CRMP-62 protein injection, when transcriptional effects are likely to be negligible, collapsin elicits a transient inward current similar to that observed after RNA injection (Fig. 2d).

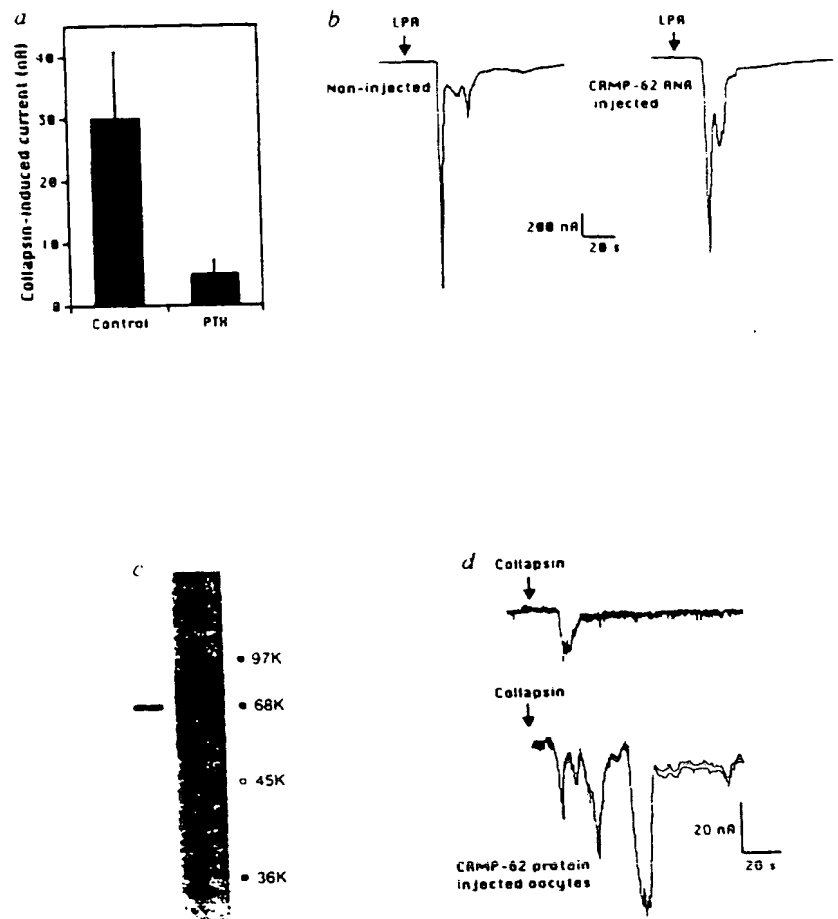
On northern blots, *CRMP-62* cDNA probe hybridizes to a 5.0 kb mRNA species in brain and DRG, but not other tissues (Fig. 3A). The expression of brain *CRMP-62* mRNA is highest at E7 and decreases by 95% in adulthood. *In situ* hybridization of E7 embryos detects high mRNA levels in the brain, retina, spinal cord and DRG (Fig. 3B). Brain levels are low in the immediate subventricular zone where primitive neuroblasts are dividing, but higher in adjacent neuronal layers of the telencephalon. Less signal is present in the developing molecular layer at the pial surface. The highest retinal levels are seen in the ganglion cell layer. Because retinal growth cones do not respond to collapsin<sup>1</sup>, CRMP-62 function may not be restricted to collapsin.

CRMP-62 protein expression was characterized with antisera directed against residues 30 to 48 or 475 to 491 of CRMP-62. Immunoblot analysis of chick E7 tissues with anti-CRMP-62(30–48) shows high levels of immunoreactivity of 62K protein in the embryonic brain and DRG (Fig. 3C), and much lower levels of immunoreactivity at 58K. Similarly, anti-CRMP-62(475–491) recognizes a 62K protein in chick embryonic DRG (Fig. 3D). In brain, trace bands between 66 and 75K are also detectable with anti-CRMP-62(30–48). Immunohistologically, CRMP-62 is detectable at DRG growth cones (Fig. 3E). CRMP-62 is abundant, comprising 0.05% of embryonic brain protein (not shown). After maximal injections of 50 ng *CRMP-62* RNA, oocytes express slightly less CRMP-62 immunoreactivity than does embryonic chick brain (not shown). Non-injected oocytes do not express detectable CRMP-62. CRMP-62 immunoreactivity is found predominantly in cytosolic fractions of embryonic brain, but 20% of immunoreactive protein remains associated with particulate fractions during extensive washing at high ionic strength and can be extracted with detergent (not shown).

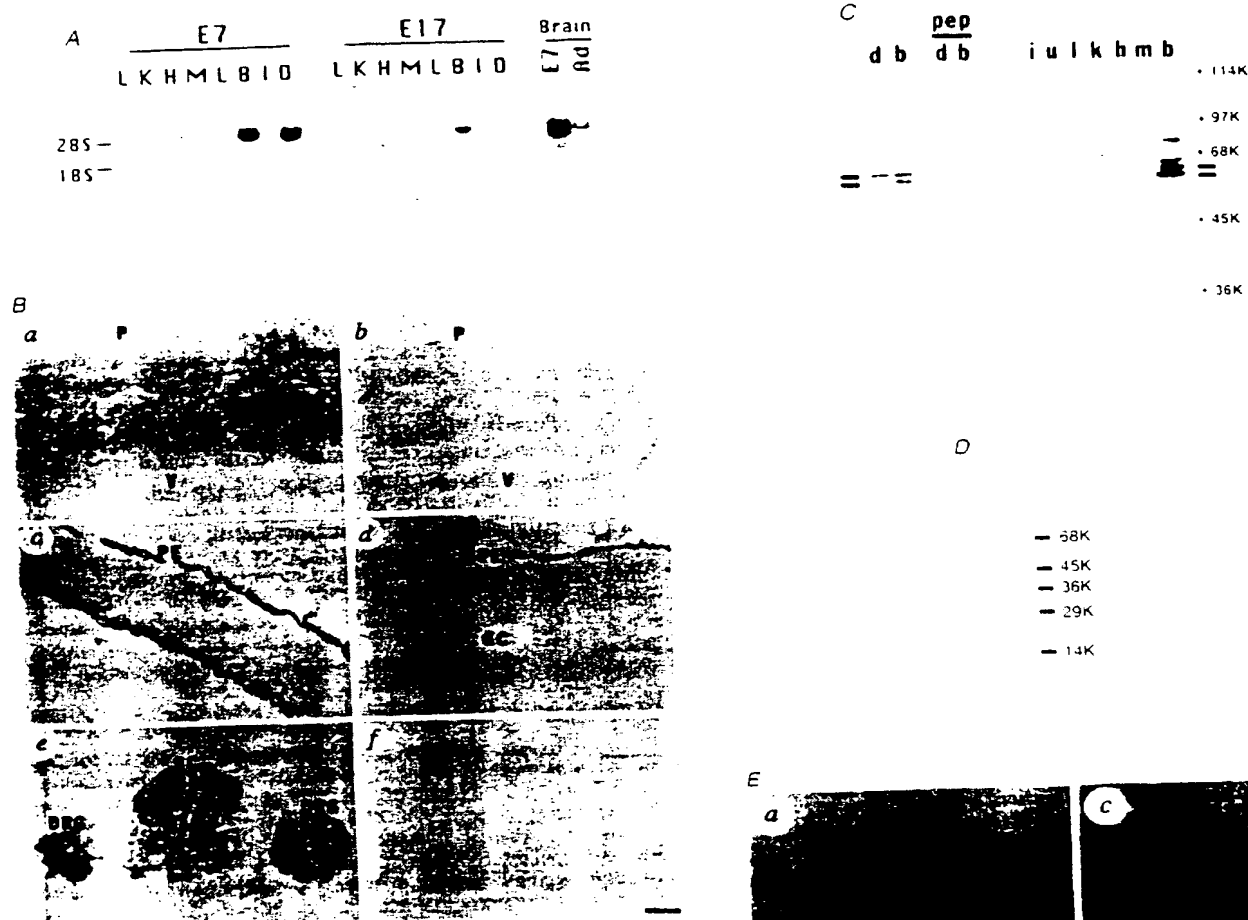
To assess the role of CRMP-62 in growth cone collapse, we used anti-CRMP-62(30–48) to block CRMP-62 function. Five minutes after injection into *CRMP-62* RNA-injected oocytes,

FIG. 2 CRMP-62 action in oocytes is PTX-sensitive, collapsin-specific and immediate. a, The effect of PTX pretreatment on the current response to recombinant collapsin in *CRMP-62* RNA-injected oocytes. PTX ( $2 \mu\text{g ml}^{-1}$ ) was added to the incubation medium 24 h before the voltage-clamp experiment. The data shown are mean inward current  $\pm$  s.e.m. for eight oocytes from the same frog in each experimental group. b, Oocyte response to lysophosphatides is not altered by injection of *CRMP-62* RNA. The inward current response after bath application of 100 nM LPA<sup>19,20</sup> in a non-injected and *CRMP-62* RNA-injected oocyte is illustrated. The average peak inward current with and without injection of 40 ng *CRMP-62* RNA was  $970 \pm 350$  nA and  $780 \pm 250$  nA, respectively ( $n=7$ ). c, A recombinant CRMP-62 fusion protein was purified from *Escherichia coli* and analysed by SDS-PAGE. The position of M<sub>r</sub> markers are shown on the right. d, Collapsin induces an electrophysiologic response in oocytes injected with recombinant CRMP-62 protein. 5 min after the injection of CRMP-62 protein, recombinant COS cell-derived collapsin was applied to the bath. Collapsin induced 20–50 nA inward currents in these two CRMP-62 protein-injected oocytes. This response is representative of 16 separate oocytes with a mean peak amplitude of  $12.5 \pm 3.6$  nA. Buffer-injected oocytes showed no detectable response ( $<2$  nA) to collapsin ( $n=16$ ). CRMP-62 protein injection alone has no detectable effect on the holding current (not shown).

**METHODS.** Electrophysiologic procedures are described in Fig. 1. CRMP-62 (20 nl of  $0.3 \text{ mg ml}^{-1}$ ) in 100 mM KCl, 10 mM Na, HEPES 0.1 mM DTT, pH 7.8 was injected into each 1,000-nl oocyte<sup>18</sup>. A bacterial expression vector encoding a fusion protein of M<sub>r</sub> 68K with 6 His residues followed by the CRMP-62 sequence was created in pTrcHis (Invitrogen). Recombinant protein was purified on a nickel-containing resin under denaturing conditions from IPTG-induced *E. coli* carrying this plasmid. Full-length CRMP-62 fusion protein was further purified by SDS-PAGE, electroelution, dialysis into 8 M urea without SDS, and then dialysis into oocyte injection buffer (10 mM NaHEPES, 100 mM KCl, 0.1 mM DTT, pH 7.8). During the extensive dialysis to remove denaturing agents, the pH was maintained above 7.7 to avoid protein aggregation.



# LETTERS TO NATURE



**FIG. 3** Localization of CRMP-62 in developing neurons. **A**, Northern blot analysis of CRMP-62 expression. Total RNA (10  $\mu$ g) from chick E7 or E17 embryonic tissues (from left: L, lung; K, kidney; H, heart; M, muscle; L, liver; B, brain; I, intestine; D, DRG) or adult (Ad) brain were hybridized to a CRMP-62 probe. The quantity of 18S and 28S ribosomal RNA was the same in each lane, and the migration of these RNAs is indicated at the left. **B**, Localization of CRMP-62 RNA in E7 chick embryo. E7 chick tissues were hybridized with an antisense (a, c, e, f) or sense (b, d) CRMP-62 probe. In the brain (a, b), CRMP-62 RNA is detected at high concentrations and is localized to deep layers of the telencephalon, but not in the region immediately adjacent to the cerebral ventricle (V). Lower levels are found in layers near the pial (P) surface. In the retina (c, d), CRMP-62 is highly expressed in the retinal ganglion cells layer (GC). The position of the pigment epithelium (PE) is indicated. In the lumbar region (e), there are high levels of CRMP-62 in the spinal cord (SC) and the dorsal root ganglia (DRG). The liver (f) and other organs (not shown) had no detectable CRMP-62 RNA. There is no staining with the sense probe (b, d). Scale bar, 60  $\mu$ m (a-d, f), or 120  $\mu$ m (e). **C**, Immunoblot analysis of CRMP-62(30-48) immunoreactivity in chick embryonic tissues. Immunoblots of total tissue homogenates containing 10  $\mu$ g protein were prepared using 0.1  $\mu$ g  $\text{ml}^{-1}$  anti-CRMP-62(30-48). The DRG (d) and brain (b) show intense immunostaining, whereas other tissues (l, intestine; u, lung; i, liver; k, kidney; h, heart; m, muscle) show very little staining. The most prominent staining is at 62K, with lesser staining at 58K (indicated by the two lines at right and left). Fainter staining is observed in brain at higher M. The middle panel illustrates the absence of staining in the presence of the CRMP-62 peptide antigen (pep). The mobilities of markers are indicated on the right. **D**, CRMP-62(475-491) immunoreactivity in chick E7 DRG. The anti-CRMP-62(475-491) antibody at 1  $\mu$ g  $\text{ml}^{-1}$  exhibits a pattern of immunoreactivity for embryonic DRG tissue similar to that in C. The mobilities of markers are shown on the right. **E**, Localization of CRMP-62 in DRG growth cone. DRG explants were fixed 24 h after plating, and immunostained with anti-CRMP-62(30-48) antibody. Note the presence of CRMP-62 immunoreactivity in neurites and growth cones (a, b). The 100  $\mu$ g  $\text{ml}^{-1}$  CRMP-62(30-48) MAP peptide (c) shows absence of staining, d shows a differential interference contrast image of the same field to illustrate the presence of growth cones. Scale bar, 20  $\mu$ m. **METHODS**. Total RNA was isolated from various tissues with acid guanidinium thiocyanate-phenol-chloroform, fractionated on 1.2% agarose-formaldehyde gels and transferred to a nylon membrane. The CRMP-62 probe was synthesized with random primed Klenow enzyme using the -88 to +633 BamHI fragment of CRMP-62 and [ $\alpha$ - $^{32}$ P]dCTP. After hybridization, the filter was washed to a final stringency of 0.1  $\times$  SSC and 0.1% SDS at 63  $^{\circ}$ C. Frozen sections (20  $\mu$ m) of chick embryos

were treated sequentially with 3.7% formaldehyde, proteinase K,  $\text{H}_2\text{O}_2$ , formaldehyde, triethanolamine and acetic anhydride. Sense and antisense digoxigenin-labelled riboprobes were prepared from clone 71 plasmid DNA. Probes (3  $\mu$ g  $\text{ml}^{-1}$ ) were hybridized to sections in 50% formamide, 5  $\times$  SSC, 50  $\mu$ g  $\text{ml}^{-1}$  yeast transfer RNA, 1% SDS and 50  $\mu$ g  $\text{ml}^{-1}$  heparin at 60  $^{\circ}$ C. After RNase A treatment, sections were washed with 50% formamide, 2  $\times$  SSC at 65  $^{\circ}$ C. Bound probe was detected using anti-digoxigenin antibody conjugated to alkaline phosphatase (Boehringer-Mannheim). Peptides corresponding to residues 30-48 (SFYADIYMEDGLIKIGEN) and 475-491 (DFYKRIKARSRLAELR) of CRMP-62 were synthesized on a branched lysine carrier core (MAP peptide, Research Genetics). Rabbits were immunized with MAP peptides and antibodies were affinity purified using an Affigel 15 resin containing 5 mg MAP peptide per ml resin. Both purified antibodies detect immobilized native recombinant CRMP-62 by ELISA at antibody concentrations above 25  $\text{ng ml}^{-1}$  (not shown). At 10  $\mu$ g  $\text{ml}^{-1}$ , both antibodies immunoprecipitate native recombinant CRMP-62 stoichiometrically (not shown). Various tissues were homogenized in 20 volumes of 10 mM Tris-HCl, 1 mM EDTA, 1 mM PMSF, 5  $\mu$ g  $\text{ml}^{-1}$  leupeptin, 1  $\mu$ g  $\text{ml}^{-1}$  pepstatin A, pH 7.8, and analysed by immunoblot with anti-CRMP-62 and peroxidase-conjugated goat anti-rabbit IgG. In blocking experiments, the CRMP-62 MAP peptide (10  $\mu$ g  $\text{ml}^{-1}$ ) or recombinant CRMP-62 protein (100  $\mu$ g  $\text{ml}^{-1}$ ) was added to the primary antibody solution (0.1  $\mu$ g  $\text{ml}^{-1}$ ) before incubation with the blot. DRG explants were cultured as in Fig. 1 for 24 h, fixed with 3.7% formaldehyde, PBS, and then incubated with 1  $\mu$ g  $\text{ml}^{-1}$  anti-CRMP-62 antibody. Bound antibody was detected using peroxidase-conjugated goat anti-rabbit IgG with diaminobenzidine as substrate.

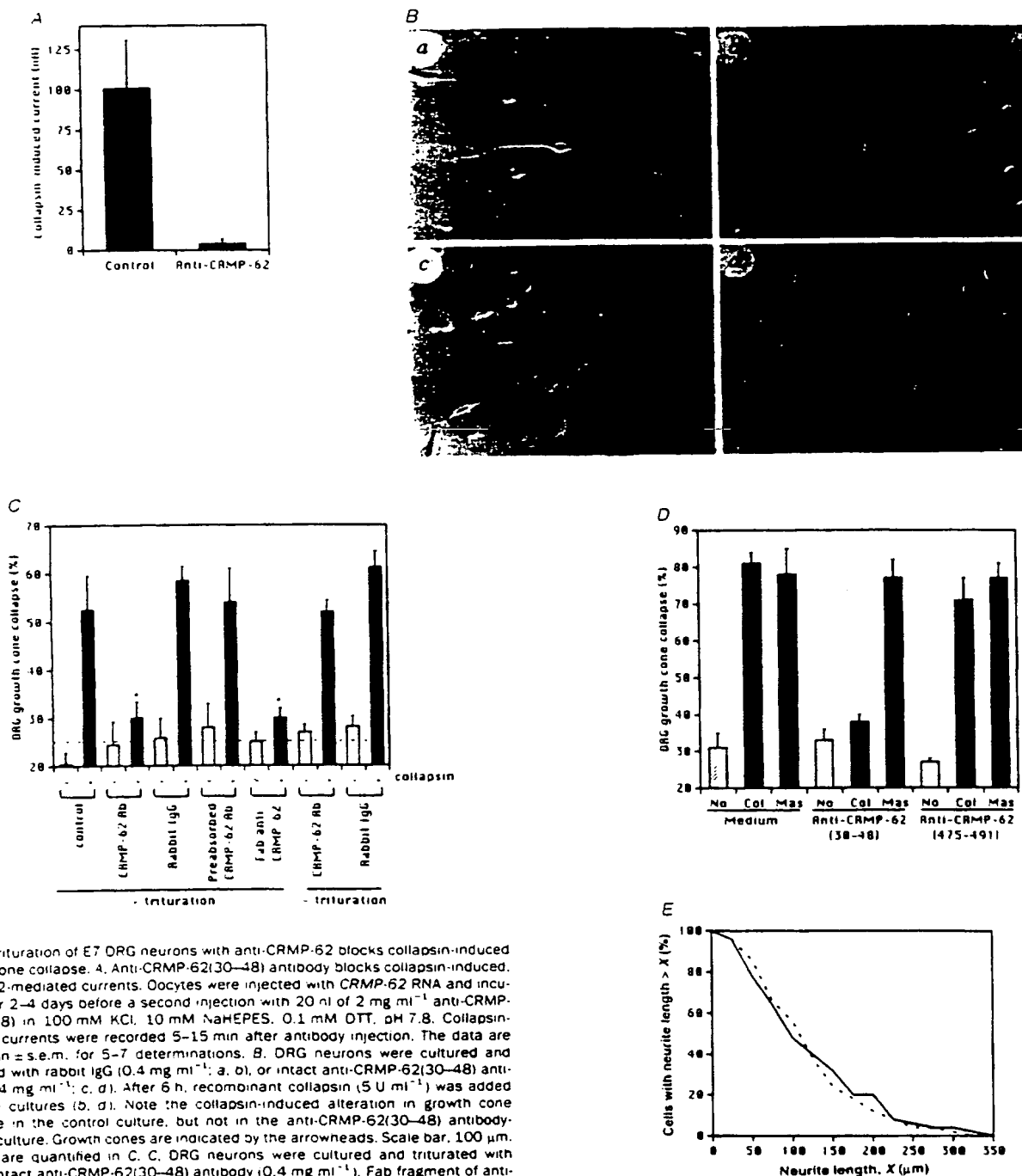


FIG. 4 Trituration of E7 DRG neurons with anti-CRMP-62 blocks collapsin-induced growth cone collapse. **A**, Anti-CRMP-62(30–48) antibody blocks collapsin-induced, CRMP-62-mediated currents. Oocytes were injected with *CRMP-62* RNA and incubated for 2–4 days before a second injection with 20 nl of 2 mg ml<sup>-1</sup> anti-CRMP-62(30–48) in 100 mM KCl, 10 mM NaHEPES, 0.1 mM DTT, pH 7.8. Collapsin-induced currents were recorded 5–15 min after antibody injection. The data are the mean  $\pm$  s.e.m. for 5–7 determinations. **B**, DRG neurons were cultured and triturated with rabbit IgG (0.4 mg ml<sup>-1</sup>; **a**, **b**), or intact anti-CRMP-62(30–48) antibody (0.4 mg ml<sup>-1</sup>; **c**, **d**). After 6 h, recombinant collapsin (5 U ml<sup>-1</sup>) was added to some cultures (**b**, **d**). Note the collapsin-induced alteration in growth cone structure in the control culture, but not in the anti-CRMP-62(30–48) antibody-treated culture. Growth cones are indicated by the arrowheads. Scale bar, 100  $\mu\text{m}$ . Results are quantified in **C**. **C**, DRG neurons were cultured and triturated with buffer, intact anti-CRMP-62(30–48) antibody (0.4 mg ml<sup>-1</sup>), Fab fragment of anti-CRMP-62(30–48) antibody (0.1 mg ml<sup>-1</sup>), rabbit IgG (0.4 mg ml<sup>-1</sup>), or preabsorbed anti-CRMP-62(30–48) antibody. The fraction of collapsed growth cones after 30 min exposure to buffer or 5 U ml<sup>-1</sup> collapsin is presented as the mean  $\pm$  s.e.m. for 4–6 separate experiments. The anti-CRMP-62(30–48) antibody and its Fab fragment significantly reduced collapsin-induced growth cone collapse (asterisk,  $P \leq 0.05$ , Student's two-tailed *t* test), whereas control solutions did not. This antibody did not alter growth cone collapse when added without trituration. **D**, DRG neurons were cultured and triturated with buffer, anti-CRMP-62(30–48) antibody (0.2 mg ml<sup>-1</sup>) or anti-CRMP-62(475–491) antibody (0.3 mg ml<sup>-1</sup>). The fraction of collapsed growth cones after 30 min exposure to buffer (No), collapsin (Col, 5 U ml<sup>-1</sup>) or mastoparan (Mas, 5  $\mu\text{M}$ ) is presented as the mean  $\pm$  s.e.m. for 6 separate experiments. The anti-CRMP-62(30–48) antibody significantly reduced collapsin-induced growth cone collapse ( $P \leq 0.05$ , Student's two-tailed *t* test), whereas the anti-CRMP-62(475–491) antibody did not. Neither antibody altered mastoparan-induced growth cone collapse. **E**, The extent of neurite outgrowth from DRG cells triturated with rabbit IgG (solid line) or the anti-CRMP-62(30–48) antibody (broken line) was determined after 5 h in culture in the absence of collapsin. The fraction of neurons with a total neurite length longer than *X* is plotted. No significant differences were detected in 4 separate experiments.

**METHODS.** Dissociated cell cultures were prepared from chick E7 DRG treated with 0.25% trypsin, 1 mM EDTA at 37 °C for 20 min<sup>8</sup>. The tissue was then triturated 30 times in growth medium using P200 plastic tips. The material was preplated on untreated plastic for 2 h to allow non-neuronal cells to attach, and then the floating cells were collected and triturated 70 times in F12 medium with 10 mM NaHEPES, pH 7.4 and various antibodies<sup>9,21</sup>. After trituration, cells were diluted eightfold into growth medium and plated on laminin-coated glass slides. Fab fragments were created using immobilized papain (Pierce) and undigested antibody was removed by protein A-agarose absorption. Immunostaining for rabbit IgG was detectable in antibody-triturated, but not control, cells (not shown). Following 6 h at 37 °C, collapsin was added to the dissociated cells and the mixture was incubated at 37 °C for 30 min before fixation. The terminus of each neurite longer than 30  $\mu\text{m}$  was scored for growth cone collapse as in the explant cultures<sup>9,8</sup>. The total outgrowth per neurite-bearing cell was quantified as described<sup>9,22</sup>. The percentage of cells bearing neurites was  $43 \pm 6\%$  in rabbit IgG-triturated cultures and  $50 \pm 6\%$  in anti-CRMP-62(30–48) antibody-triturated cultures (mean  $\pm$  s.e.m.,  $n = 4$ ).

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the antibody blocks collapsin-induced currents (Fig. 4A). Trituration of DRG neurons with anti-CRMP-62(30-48) blocks subsequent collapsin-induced growth cone collapse (Fig. 4B, C). Rabbit IgG and preabsorbed anti-CRMP-62(30-48) antibody have no effect. Addition of anti-CRMP-62(30-48) after trituration does not prevent collapsin-induced collapse. The Fab fragment of anti-CRMP-62(30-48) is as effective as intact immunoglobulin (Fig. 4C), indicating that cross-linking and aggregation of antigen do not account for these effects. Trituration with anti-CRMP-62(30-48) has no effect on neuronal survival, neurite formation or extension in the absence of collapsin (Fig. 4E).

We also trituated cells with anti-CRMP-62(475-491). This portion of CRMP-62 is not conserved with UNC-33 or between hCRMP-1 and hCRMP-2, and is not included in the original truncated clone isolated by oocyte expression. The anti-CRMP-62(475-491) antibody does not block collapse induced by collapsin or mastoparan (Fig. 4D). This verifies that the 475-491 region is not critical for CRMP-62 function, and that blockade of collapsin action by anti-CRMP-62(30-48) cannot be explained as a nonspecific effect of antibody colocalization with CRMP-62. Mastoparan stimulates PTX-sensitive G proteins and induces growth cone collapse in control cultures<sup>8,9</sup> and those trituated with anti-CRMP-62(30-48) antibody (Fig. 4D). Thus, those elements of the collapse pathway which are downstream of G protein activation are not disrupted by the anti-CRMP-62(30-48) antibody.

The hypothesis that collapsin activates a G protein signalling cascade is based on several findings: pertussis holotoxin blocks collapsin effects; collapsin activates an oocyte  $\text{Ca}^{2+}$ -sensitive chloride channel; and G protein activation collapses growth cones. CRMP-62 is likely to mediate or facilitate an interaction between a collapsin-binding transmembrane receptor and intracellular G proteins. In such a model, CRMP-62 may be an intracellular component of a multisubunit receptor, or may act as an intermediary between receptor and G protein. CRMP-62 cannot act downstream of the G protein because the response to ligands for other G protein-coupled receptors is not altered by CRMP-62, and because mastoparan-induced collapse is not blocked by anti-CRMP-62(30-48).

Generally, G protein-coupled receptors contain seven membrane-spanning domains and couple directly to G proteins. A collapsin-binding protein has not yet been identified, but its apparent requirement for CRMP-62 and its ability to respond to a macromolecular ligand which can exist in membrane-bound forms<sup>1-4</sup> imply that it has unique characteristics. Whether CRMP-62 and/or a collapsin-binding protein interact directly or indirectly with a G protein remains to be determined. It is clear that CRMP-62 is expressed selectively in developing nervous system, is capable of reconstituting a collapsin-regulated G protein cascade in oocytes, and is essential for collapsin-induced DRG growth cone collapse. Further analysis of the protein-protein interactions of CRMP-62 should lead to a more complete understanding of the molecular mechanism whereby repulsive axon guidance clues are transduced into changes in growth cone motility. □

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## Channel gating governed symmetrically by conserved leucine residues in the M2 domain of nicotinic receptors

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Nicotinic acetylcholine receptors (nAChR), as well as glycine, GABA<sub>A</sub> ( $\gamma$ -aminobutyric acid), serotonin (5-HT<sub>3</sub>), and GluCl glutamate receptors, a leucine residue at the approximate midpoint of the M2 transmembrane domain (the 9' position<sup>1</sup>) is conserved across most known subunits<sup>2</sup>. Structural data for the nAChR suggest that the Leu 9' residues occupy a 'kink' in each of the five M2 helices and point into the closed channel; in the opening step, the M2 helices rotate so that Leu 9' side chains no longer occlude the conduction pathway<sup>3</sup>. Mutation of Leu 9' to one of several other residues slows desensitization and increases sensitivity to agonist<sup>4-6</sup>. We have exploited the  $\alpha\beta\gamma\delta$  stoichiometry of muscle nAChR to express receptors with  $m_2^* = 0$  to 5 Leu 9'/Ser mutated subunits. Strikingly, each Leu 9'/Ser mutation shifts the dose-response relation for ACh to the left by ~10-fold: a nAChR with  $m_2^* = 4$  is 10-fold more sensitive than the wild type. The results suggest that each of the five Leu 9' residues participates independently and symmetrically in a key step in the structural transition between the closed and open states.


Oocytes were injected with mRNA for wild-type subunits or for subunits containing the Leu 9'/Ser mutation; the latter are denoted here with an asterisk. Figure 1 shows raw traces and dose-response data for nAChRs that exemplify combinations for  $m_2^* = 1, 2, 3$  and 4 Leu 9'/Ser mutations, respectively. The clear trend is that receptors with increasing  $m_2^*$  require decreased ACh concentrations for half-maximum response ( $EC_{50}$ ). We analysed these dose-response relations for all four possible hetero-oligomeric combinations with  $m_2^* = 1$  and for all but one of the combinations yielding  $m_2^* = 2, 3, 4$  and 5 that contain either 2 $\alpha$  or 2 $\alpha^*$  subunits (Table 1; the  $\alpha^*\beta^*\gamma^*\delta$  combination gave no detectable expression). The  $EC_{50}$  for ACh decreases by ~10-fold for each additional mutated subunit (Fig. 1c). Thus the wild-type nAChR has an  $EC_{50}$  of 24  $\mu\text{M}$ , and the two combinations tested with  $m_2^* = 4$  have  $EC_{50}$  of 2.0 and 2.3 nM.

There is a range of up to fourfold among the  $EC_{50}$  values within some groups with equal  $m_2^*$ . These extrema were not consistently associated with a particular mutated subunit.

Dose-response relations for the  $\alpha^*\alpha\beta\gamma\delta$  receptor (in the  $m_2^* = 1$  group) were determined by injecting oocytes with mixtures of  $\alpha$  and  $\alpha^*$  messenger RNA, along with  $\beta, \gamma$  and  $\delta$  (for example,

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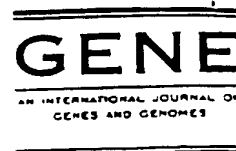
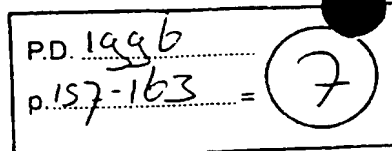
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551 PRRTGHRIVA PPGGRSNITS LG





XP 002045121

Gene 180 (1996) 157–163



# A novel gene family defined by human dihydropyrimidinase and three related proteins with differential tissue distribution

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## Abstract

We have isolated cDNA clones encoding dihydropyrimidinase (DHPase) from human liver and its three homologues from human fetal brain. The deduced amino acid (aa) sequence of human DHPase showed 90% identity with that of rat DHPase, and the three homologues showed 57–59% aa identity with human DHPase, and 74–77% aa identity with each other. We tentatively termed these homologues human DHPase related protein (DRP)-1, DRP-2 and DRP-3. Human DRP-2 showed 98% aa identity with chicken CRMP-62 (collapsin response mediator protein of relative molecular mass of 62 kDa) which is involved in neuronal growth cone collapse. Human DRP-3 showed 94–100% aa identity with two partial peptide sequences of rat TOAD-64 (turned on after division, 64 kDa) which is specifically expressed in postmitotic neurons. Human DHPase and DRPs showed a lower degree of aa sequence identity with *Bacillus stearothermophilus* hydantoinase (39–42%) and *Caenorhabditis elegans unc-33* (32–34%). Thus we describe a novel gene family which displays differential tissue distribution: i.e., human DHPase, in liver and kidney; human DRP-1, in brain; human DRP-2, ubiquitously expressed except for liver; human DRP-3, mainly in heart and skeletal muscle.

**Keywords:** Amidohydrolase; cDNA cloning; CRMP-62; Dihydropyrimidine; TOAD-64; *unc-33*

## 1. Introduction

Dihydropyrimidinase (5,6-dihydropyrimidine amido-hydrolase (EC 3.5.2.2) DHPase) is the second enzyme involved in uracil and thymine catabolism. DHPase catalyzes the hydrolysis of 5,6-dihydrouracil to *N*-carbamyl-β-alanine and 5,6-dihydrothymine to *N*-carbamyl-β-aminoisobutyrate (Fritzson, 1957; Fritzson and Phil, 1957). DHPase also catalyzes the hydrolysis of a variety of 5,6-dihydropyrimidines (Wallach and Glisolia, 1957;

Kim et al., 1976) as well as hydantoins and succinimides (Dudley et al., 1974; Maguire and Dudley, 1978). DHPase is a tetrameric Zn<sup>2+</sup>-metalloenzyme, containing four tightly bound Zn ions/molecule of active enzyme (Brooks et al., 1979, 1983; Kikugawa et al., 1994). The DHPase which we purified from rat liver has a molecular mass of 215 kDa and a subunit mass of 54 kDa (Kikugawa et al., 1994). Recently, we isolated a cDNA clone encoding DHPase (DHP) from rat liver, which contained a 1560-bp open reading frame (ORF) encoding a polypeptide of 519 residues (Matsuda et al., 1996). The sequence of this rat DHPase exhibited approximately 40% amino acid (aa) identity with those of hydantoinase from *Pseudomonas putida* and *Bacillus stearothermophilus* (Lapointe et al., 1994; Mukohara et al., 1994). We report here the cloning and structural analysis of human liver DHP cDNA which we undertook to investigate dihydropyrimidinuria (McKusick 222748) (Duran et al., 1990; Henderson et al., 1993; Ohba et al., 1995) at the molecular level.

During the cloning of human DHP cDNA, we noted

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Abbreviations: aa, amino acid(s); bp, base pair(s); cDNA, DNA complementary to RNA; CRMP-62, collapsin response mediator protein of relative molecular mass 62 kDa; DHPase, dihydropyrimidinase; DHP, gene (DNA) encoding DHPase; DRP, dihydropyrimidinase related protein; DRP, gene (DNA) encoding DRP; EST, expressed sequence tag; kb, kilobase(s) or 1000 bp; kDa, kilodalton; *M<sub>r</sub>*, relative molecular mass (dimensionless); ORF, open reading frame; RT-PCR, reverse transcriptase-polymerase chain reaction; TOAD-64, turned on after division, 64 kDa; UTR, untranslated region(s).

a series of sequences homologous to human *DHP* in the DDBJ, EMBL and GenBank data bases. Two sequences thought to be related to nervous system development showed a high degree of aa identity: chicken CRMP-62 (collapsin response mediator protein of relative molecular mass 62 kDa), which is required for collapsin-induced growth cone collapse (Goshima et al., 1995), and rat TOAD-64 (turned on after division, 64 kDa), which is exclusively expressed by postmitotic neurons as they begin their migration out of the ventricular zone into the developing cortical plate (Minturn et al., 1995). In addition, human *DHPase* showed a lower degree of aa identity with the putative product of the shortest cDNA species of *Caenorhabditis elegans unc-33*, which was identified by a mutation resulting in severely uncoordinated movement, abnormalities in the guidance and outgrowth of axons of many neurons, and a superabundance of microtubules in neuronal processes (Li et al., 1992). Moreover, a number of human expressed sequence tags (ESTs) mainly from brain showed 63-71% nucleotide (nt) identity with human *DHP*. Sequence comparison of the overlapping regions of these human ESTs revealed the presence of three distinctive, but highly similar sequences which are considered to form a gene family together with human *DHP*. As the first step in elucidating the structural and functional aspects of this gene family, we isolated three types of cDNA clones from human fetal brain whose products we termed human *DHPase* related protein (DRP)-1, DRP-2 and DRP-3. The deduced aa sequences of human *DHPase* and DRPs as well as their differential tissue distribution are presented.

## 2. Materials and methods

### 2.1. Isolation of cDNA encoding human *DHPase*

RT-PCR amplification of a portion of the rat *DHP* sequence has already been described (Matsuda et al., 1996). Using the PCR amplified cDNA fragment as a probe, a human adult liver cDNA library (Stratagene) was screened. Twenty-five positive clones were isolated and converted into pBluescript SK(+) plasmid. The clone with the largest insert of approximately 2.2 kb was completely sequenced using a 373A DNA sequencer (Applied Biosystems). Each sequence was determined from both strands.

### 2.2. Isolation of cDNAs encoding three human DRPs

A human fetal brain cDNA library (17 weeks) was constructed using a cDNA Synthesis System Plus (Amersham), an *EcoRI* Adapter (Promega), a Lambda ZAP II Vector (Stratagene) and Gigapack II Gold Packaging Extract (Stratagene). To obtain probes for

screening, human fetal brain cDNA was amplified by RT-PCR with primers derived from the nt sequences of human ESTs from brain and mouse *Ulip* (EMBL accession No. X87817), a putative mouse counterpart of human *DRP-3*. For human *DRP-1* cDNA, the sense primer was 5'-GTGGGCGGGACCACGATGAG-3' (from GenBank accession No. T06278) and the antisense primer was 5'-AATGGGCTCTCCAAAACTA-3' (from GenBank accession No. F11378). For human *DRP-2* cDNA, the sense primer was 5'-CCTTAAAGCTGCCCTCTTGA-3' (from GenBank accession No. T07524) and the antisense primer was 5'-GATGGTGATGGCTCGATTCA-3' (from DDBJ accession No. D38743). For human *DRP-3* cDNA, the sense primer was 5'-GCCATTGGGAAGGACAACCTT-3', corresponding to the nt sequence of mouse *Ulip* at positions 1333-1352, and the antisense primer was 5'-TGGAGGAAGGCTTGCTTAACCT-3' (from GenBank accession No. T05158). The PCR products of 545, 769 and 704 bp, respectively, were obtained and subcloned into the pCR II vector (Invitrogen). Each cDNA fragment was sequenced using a 373A DNA sequencer (Applied Biosystems). Using the PCR amplified cDNA fragments as probes, approximately 400 000 plaques of the human fetal brain cDNA library were screened. Approximately 200 plaques for human *DRP-1*, 30 for *DRP-2* and 30 for *DRP-3* were identified at the first screening, and 19, 10 and 19 positive clones, respectively, were isolated and converted into pBluescript SK(+) plasmid. The clones with the largest inserts of approximately 2.8 kb for *DRP-1*, 4.5 kb for *DRP-2* and 5.1 kb for *DRP-3* were completely sequenced using the 373A DNA sequencer (Applied Biosystems). Each sequence was determined from both strands.

### 2.3. Northern blotting analysis

A Human Multiple Tissue Northern Blot (CLONTECH) was hybridized with <sup>32</sup>P-labelled DNA probes synthesized using the Rediprime DNA labelling system (Amersham). An 878-bp *Pst*I-digested fragment from the 5'-region of the human *DHP* cDNA clone was used as a probe for Northern blotting analysis of human *DHP*. Three probes used for the screening of three human *DRP* cDNAs were also used as probes for Northern blotting analysis of three human *DRPs*. Hybridization was performed in 5 × SSPE (0.75 M NaCl, 0.05 M NaH<sub>2</sub>PO<sub>4</sub> and 5 mM EDTA), 10 × Denhardt's solution (0.2% Ficoll, 0.2% poly(vinylpyrrolidone) and 0.2% bovine serum albumin), 100 µg ml<sup>-1</sup> salmon sperm DNA, 50% deionized formamide and 2% sodium dodecyl sulfate (SDS) for 18 h at 42°C, and the filters were washed twice in 0.1 × SSC (15 mM NaCl and 1.5 mM Na citrate) and 0.1% SDS for 30 min at 65°C.



## 2.4. Computer analysis

Homology searches of the DDBJ, EMBL and GenBank data bases were performed using the BLAST network service. Alignment of aa sequences and construction of the phylogenetic tree were performed using the CLUSTAL W program (Thompson et al., 1994).

## 3. Results and discussion

### 3.1. Isolation and sequence analysis of human DHP cDNA

The human *DHP* cDNA clone with an insert of 2129 bp contained a 116-bp 5'-untranslated region (*UTR*), a 1560-bp ORF (including the stop codon) encoding a polypeptide of 519 aa with a predicted  $M_r$  of 56 629 and a 444-bp 3'-*UTR* including a polyadenylation signal (AATAAA) 25 bp upstream from a 9-bp poly(A) tail. The aa sequence from this clone showed 90 and 42% identity with rat DHPase and *B. stearothermophilus* hydantoinase, respectively. Homology searches of the DDBJ, EMBL and GenBank data bases using the BLAST network service revealed the presence of a series of sequences homologous to human DHPase (Table 1). Moreover, a number of human ESTs, mainly from brain, showed 63–71% nt identity with human DHPase.

### 3.2. Isolation and sequence analysis of three human DRP cDNAs

Three distinctive cDNA species were isolated from a human fetal brain cDNA library, and the clones of each species with the largest inserts were sequenced entirely. The human *DRP-1* clone with an insert of 2842 bp contained a 150-bp 5'-*UTR*, a 1719-bp ORF (including the stop codon) encoding a polypeptide of 572 aa with a predicted  $M_r$  of 62 182, and a 973-bp 3'-*UTR* including a polyadenylation signal (AATAAA) 16 bp upstream from its 3'-end. The human *DRP-2* clone with an insert of 4507 bp contained a 274-bp 5'-*UTR* which included an inframe stop codon, a 1719-bp ORF (including the stop codon) encoding a polypeptide of 572 aa with a predicted  $M_r$  of 62 292, and a 2466-bp 3'-*UTR* including a polyadenylation signal (AATAAA) 19 bp upstream from a 48-bp poly(A) tail. The human *DRP-3* clone with an insert of 5124 bp contained a 110-bp 5'-*UTR* which included an inframe stop codon, a 1713-bp ORF (including the stop codon) encoding a polypeptide of 570 aa with a predicted  $M_r$  of 61 962 and a 3224-bp 3'-*UTR* including an atypical polyadenylation signal (AAATAA) 12 bp upstream from a 77-bp poly(A) tail.

Alignment of aa sequences of human DHPase, three human DRPs (Fig. 1), and related sequences of other species (data not shown), was performed. There was a high degree of aa identity over the entire coding regions among these sequences, except for their C-terminal end.

Table 1  
The percent aa identity among DHP gene family members<sup>a</sup>

	%Identity										
	Hu DRP-1	Hu DRP-2	Ra TOAD-64 <sup>b</sup>	Mo <i>unc-33</i> <sup>c</sup>	Ch CRMP-62 <sup>d</sup>	Hu DRP-3	Mo Ulip <sup>e</sup>	Hu DHPase	Ra DHPase <sup>f</sup>	Bs hydantoinase <sup>g</sup>	Ce <i>unc-33</i> <sup>h</sup>
Hu DRP-1	100	76	76	76	78	74	74	57	56	40	32
Hu DRP-2		100	99	98	98	77	76	59	57	39	33
Ra TOAD-64 <sup>i</sup>			100	99	97	77	76	59	57	39	32
Mo <i>unc-33</i>				100	97	77	76	59	57	39	32
Ch CRMP-62					100	77	77	59	58	40	33
Hu DRP-3						100	98	58	57	40	33
Mo Ulip							100	57	56	40	33
Hu DHPase								100	90	42	34
Ra DHPase									100	42	33
Bs hydantoinase										100	27
Ce <i>unc-33</i>											100

<sup>a</sup>The % aa identity was calculated based on the alignment carried out using the CLUSTAL W program.

<sup>b</sup>This nt sequence predicted a similar but distinct aa sequence with two partial peptide sequences of rat TOAD-64 which were reported previously (Minturn et al., 1995). Therefore, we tentatively designated this nt sequence as rat TOAD-64<sup>i</sup> (EMBL accession No. Z46882).

<sup>c</sup>Mouse *unc-33* (EMBL accession No. X87242).

<sup>d</sup>Chicken CRMP-62 (GenBank accession No. U17277).

<sup>e</sup>Mouse Ulip (EMBL accession No. X87817).

<sup>f</sup>Rat DHPase (DDBJ accession No. D63704).

<sup>g</sup>*B. stearothermophilus* hydantoinase (GenBank accession No. S73773).

<sup>h</sup>*C. elegans unc-33* (EMBL accession No. Z14146).

Hu DRP-1	MSYQGKKSIPHITSDRLLIKGGRIINDDOQSLYADVLYEDGLIKQIGENLIVPG----	GVK	56
Hu DRP-2	.....N..R.....K.V....F...I.M.....		56
Hu DRP-3	.....N..R.....V....F...I.M.....D.....		56
Hu DHPase	MAAPS....R...VV...F.EV...LV...VVRAL.HD.LP..GAPA.LR		50
Hu DRP-1	TIEANGRMVIPGGIDVNTYLQKPSQGMATAODFFQGTAAALVGGTTMIIDHVVPFGSSSL		116
Hu DRP-2	....HS.....H.RF.M.D....S.....K...A.....T..		116
Hu DRP-3	.....K.....H.HF.M.YK...TV.....K...A.....E...		116
Hu DHPase	VLD.A.KL.L....TH.HM.F.FM.SRSI...H...K...S.....FAI.QK.G..		110
Hu DRP-1	LTSFEKWHEAADTKSCCDYSLHVDITSWYDGVREELEVLVQDKGVNSFQVYMAKYDVYQM		176
Hu DRP-2	..AA.DQ.R.W..S.....SE.BK.IQ..M.A..K..H.....L....F...RF.L		176
Hu DRP-3	TEAY...R.W..G.....A.....H.N.S.KQ.VQN.IK.....M.....L..V		176
Hu DHPase	IEA..T.RSW..P.V.....AV.W.S.Q.K..MKI.....KMF.....L.MV		170
Hu DRP-1	SDSQLYEAFTFLKGLGAVILVHAENGDLIAQEQKRILEMGITGPEGHALSRPEELEAEAV		236
Hu DRP-2	T.C.I..VLSVIRDI..IAQ.....I..E..Q...DL.....V.....V.....		236
Hu DRP-3	.NTE...I..C.GE...IAQ.....I.....T.M.....V.....		236
Hu DHPase	T.LE....SRC.EI..IAQ.....EGA.KM.AL.....E.C...AV....T		230
Hu DRP-1	FRAITIAGRINCPVYITKVMKSAAIIALARKKGPLVFGPEPIAASLGTGDGTHYWSKNWA		296
Hu DRP-2	N.....NQT...L.....S.EV..Q....TV.Y....T.....S.....		296
Hu DRP-3	.....SQT...L.V.....L.SQ....NV.....T...I.....		296
Hu DHPase	L.....SAV...L..VH.....KV..D..RD.KV.Y.....N.E.H		290
Hu DRP-1	KAAAFVTSPPLSPDPTTPDYLTSLACGDLQVTGSGHCPYSTAQKAVGKDNFTLIPEGVN		356
Hu DRP-2	.....F.N...S.....A..TFN.....T..		356
Hu DRP-3	.....IN...S....LS..A..TF.....I.....A...T..		356
Hu DHPase	H..HH.MG...R...S...F.MN...ND..TT..TDN.TFN.C...L...D..K..N...		350
Hu DRP-1	GIEERMTVVWDKAVATGKMNDENQFVAVTSTNAAKIFNLVPRKGRIAGVSDADVVIWDPDK		416
Hu DRP-2	.T....S.I.....V.....V.....L.....S		416
Hu DRP-3	.V....S.I.....S.....S.L.....A		416
Hu DHPase	.V.D..S.I.E.G.BS.....R.....I.....KG		410
Hu DRP-1	LKTITAKSHKSAVEYNIFEGMECHGSPVVISQKIVFEDGNINVNKGMRFPKAFPE		476
Hu DRP-2	V...S...T.N.SL.....R.....L...TLH.TE.S..Y...P..D		476
Hu DRP-3	V.IVS..N.Q..A.....LR.A....C...ML...LH.TQ.A....CSP.SD		476
Hu DHPase	TR..S..T.HQ..NF.....V...V...T..R..V.Y.A.VFS.TA.D.K....P.A.		470
Hu DRP-1	HLVQVRVKIRNKVFLQGVSRGMYDGPVYEVPAATPKYATPAPSAKSSPSKHQPPPIRNLEQ		536
Hu DRP-2	FV.K.I.A.SRLAE.R..P..L....C..SV...TV...S...T..A.Q.A..V.....		536
Hu DRP-3	YV.K.I.A.R.MAD.HA.P.....FDLTT...GG...G..RG..TRPN...V.....		535
Hu DHPase	YI.K.I.Q.DRTCTPTP.E.AP.K.E.ATLKSrv---TKEDA-TAGTRKQAH.		519
Hu DRP-1	SNFSLSGAQIDDNNPRRTGHRIVAPPGGRSNTSLG		572
Hu DRP-2	.G.....I...TQ.....A.....		572
Hu DRP-3	.G.....T.V.EG-V.SASK.....S		570

Fig. 1. Alignment of the aa sequences of human DHPase and three human DRPs. The alignment of human DHPase (Hu DHPase), human DRP-1 (Hu DRP-1), human DRP-2 (Hu DRP-2) and human DRP-3 (Hu DRP-3) was performed using the CLUSTAL W program. Identical residues with human DRP-1 in human DRP-2, DRP-3 and DHPase are shown by dots. Gaps introduced to increase the identity are shown by dashes. The nt sequence data of human DHPase, human DRP-1, DRP-2 and DRP-3 will appear in the DDBJ, EMBL and GenBank nt sequence databases with accession Nos. D78011, D78012, D78013 and D78014, respectively.

The calculated percentages of aa identity among these sequences based on this alignment are shown in Table 1. In addition, the aa sequence of DRP-3 showed 94–100% identity with two partial peptide sequences of rat TOAD-64. From these results, we concluded that human DRP-2 is a counterpart of rat TOAD-64, mouse *unc-33* and chicken CRMP-62; and human DRP-3, a counter-

part of mouse Ulip and rat TOAD-64. A lower degree of aa identity was observed between these sequences and *B. stearotheophilus* hydantoinase (39–40%) or *C. elegans unc-33* (32–33%). A phylogenetic tree of the DHPase related sequences was constructed based on this alignment using the neighbor-joining method (Saitou and Nei, 1987) and is shown in Fig. 2. The *C. elegans unc-33*

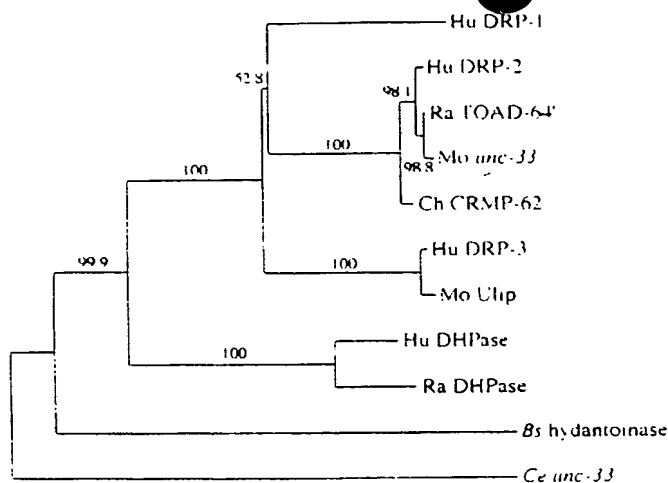


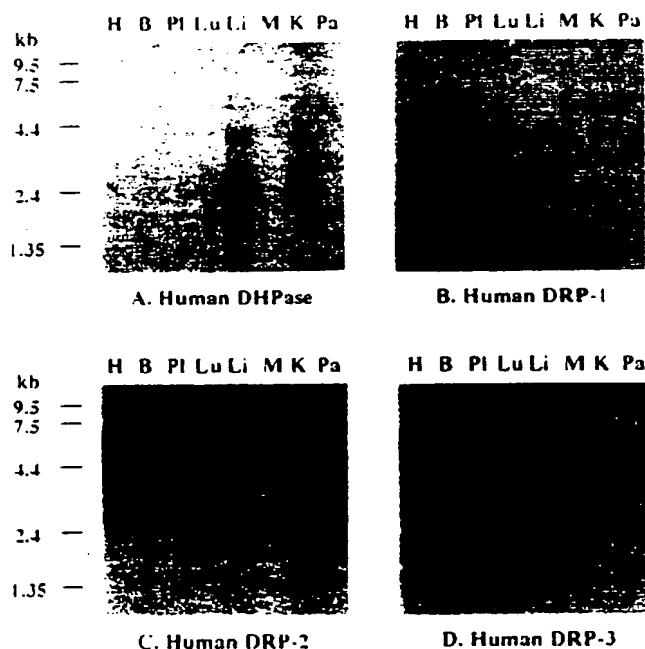
Fig. 2. Phylogenetic tree of *DHP* gene family. The relationship among human DHPase (Hu DHPase), rat DHPase (Ra DHPase), human DRP-1 (Hu DRP-1), human DRP-2 (Hu DRP-2), human DRP-3 (Hu DRP-3), rat TOAD-64 (Ra TOAD-64), mouse *unc-33* (Mo *unc-33*), chicken CRMP-62 (Ch CRMP-62), mouse Ulip (Mo Ulip), *B. stearothermophilus* hydantoinase (*Bs* Hydantoinase) and *C. elegans unc-33* (*Ce unc-33*) was analyzed by the CLUSTAL W program. The phylogenetic tree was constructed by the neighbor-joining method based on this alignment. Numbers on branches are bootstrap percentages supporting a given partitioning.

sequence shows the greatest divergence from the other sequences compared here. The next most divergent sequence is that of *B. stearothersophilus* hydantoinase, and human and rat DHPase which form a group which diverged from three human DRPs and their counterparts in other species. Thus, mammalian DHP, mammalian and avian DRP, the gene encoding *B. stearothersophilus* hydantoinase and *C. elegans unc-33* form a novel gene family, which we have tentatively termed the DHP gene family.

Partial sequences for human *DRP-1* and *DRP-2* have been reported as hCRMP-1 (GenBank accession No. U17278) and hCRMP-2 (GenBank accession No. U17279), respectively, which were identified by sequence analysis of EST clones homologous to CRMP-62 (Goshima et al., 1995). However, the position of the start codon of hCRMP-1 has been assigned to the 64th aa of the human *DRP-1* sequence, probably because the EST clone used for hCRMP-1 sequence analysis was truncated at its 5'-end. Our assignment of this codon is based on the facts that: (i) this is the most upstream start codon in the sequence; (ii) GCC at the relative nt position of -3 to -1 matches Kozak's consensus sequence (Kozak, 1986); and (iii) when aligned with human *DRP-2*, *DRP-3* and their counterparts in other species, the position of this Met corresponds with the position of the start Met of other sequences (Fig. 1).

### 3.3. Differential tissue distribution of human DHase and three human DRPs

The distribution of human *DHP* and *DRP* mRNAs in eight different adult organs was examined by Northern blotting using a Human Multiple Tissue Northern Blot (CLONTECH), as shown in Fig. 3. Hybridization and washing were carried out at high stringency to prevent cross-hybridization. Human *DHP* mRNA was detected at a very high level in liver and at a high level in kidney as two bands, a major 2.5-kb band and a minor 3.8-kb band (Fig. 3A). Human *DRP-1* mRNA was detected at a high level in brain as two bands, a major 2.9-kb band and a minor 4.9-kb band (Fig. 3B). Human *DRP-2* mRNA was detected at a very high level in brain, at a high level in heart and lung, and at a low level in placenta, skeletal muscle, kidney and pancreas as a 4.9-kb band (Fig. 3C). Human *DRP-3* mRNA was detected at a high level in heart and skeletal muscle, and at a low level in brain and lung as a 5.8 kb-band (Fig. 3D). The size of the major band of each mRNA was in good agreement with that of the longest insert of each cDNA clone. Thus, human *DHP* and *DRP* mRNAs showed differential tissue distribution. Interestingly, tissue distribution patterns of *DRP* mRNAs showed an



**Fig. 3.** Northern blotting analysis of human *DHP* and three human *DRPs*. A Human Multiple Tissue Northern Blot (CLONTECH) was hybridized according to the manufacturer's instructions with  $^{32}$ P-labelled DNA probes synthesized by Rediprime DNA labelling system (Amersham). Hybridizations were carried out with probes for human *DHP* (A), human *DRP-1* (B), human *DRP-2* (C) and human *DRP-3* (D). Each lane contained 2  $\mu$ g poly(A)<sup>+</sup> RNA from human adult heart (H), brain (B), placenta (Pl), lung (Lu), liver (Li), skeletal muscle (M), kidney (K) and pancreas (Pa).

apparent discrepancy among species. Although human *DRP-2* is widely expressed in various adult tissues except for liver, its chicken counterpart, CRMP-62, is reported to be brain specific in E7 and E17 embryo (Goshima et al., 1995). Moreover, whereas human *DRP-3* is mainly expressed in heart and skeletal muscle, its possible rat counterpart, TOAD-64, is expressed in a brain-specific manner in the P1 neonate (Minturn et al., 1995). On the other hand, in human adult tissues, *DRP-1* with no known counterparts in other species, shows a brain-specific expression. This apparent discrepancy may reflect a difference in the developmental stage of the tissue source. Our data were obtained using adult tissues, whereas other investigators have used embryos or neonates. Further studies involving isolation of counterparts from various experimental animals as well as analysis of tissue distribution of human *DRP* mRNAs among fetal tissues, will resolve this apparent discrepancy.

### 3.4. Possible chromosomal localization of human *DRP* genes

There are some ESTs homologous to human *DRPs* which have a defined chromosomal localization. D25269 (DDBJ accession number) which corresponds to a portion of the 3'-UTR of human *DRP-1* cDNA is located at 4p15-16.1, near the Huntington disease loci (Ishida et al., 1994). D38661 (DDBJ accession number), D38742 (DDBJ accession number) and D38743 (DDBJ accession number) which correspond to a portion of the coding region of human *DRP-2* cDNA are localized at 8p21 (Koyama et al., 1995). However, Z47338 (EMBL accession number) which also corresponds to a portion of the coding region of human *DRP-2* cDNA is localized on chromosome 21. Regardless of the location of the actual locus for the human *DRP-2* gene, the human *DRP-1* and *DRP-2* genes seem to reside on different chromosomes.

### 3.5. Functional implication of three human *DRPs*

Although some of the human *DRP* counterparts in other animals and *C. elegans* have been shown to be involved in development of the nervous system, the biochemical basis for their function remains to be clarified. The data presented here demonstrates that the primary structure of DHPase has a higher degree of similarity to DRPs than to bacterial hydantoinase, suggesting the intriguing possibility that DRPs may also be varieties of amidohydrolase. We could not detect, however, DHPase activity in extra-hepatic tissues except for kidney in adult and newborn rats (Tamaki et al., unpublished observation), and furthermore recombinant CRMP-62 did not show hydantoinase activity (Goshima et al., 1995). Therefore, DRPs may act on substrates other than DHPase or hydantoinase, even if they are

actually amidohydrolases. In this context, it is noteworthy that there is a highly conserved region among the DHPase and DRPs sequences. As shown in Fig. 1, the 42-aa stretch (from the 373rd to 414th residue of human DHPase) is completely conserved among these sequences, except for only five positions where conservative aa substitutions were observed. Although a high degree of conservation implies functional importance, this region is not required for CRMP-62, since the original functionally isolated CRMP-62 clone lacked this region. This may imply that DRPs, and probably DHPase as well, are multifunctional proteins which are capable of both amidohydrolase activity as well as other types. As shown in Table 1, the human and rat DHPase aa sequences show 90% identity, whereas DRPs show higher conservation, 97–99%, among mammalian species or even between mammalian and avian species. It is, therefore, suggested that DRPs are involved in a basic cellular process. Together with what we know about the wide tissue distribution of *DRP* mRNA, it seems probable that *DRP* is not restricted to functioning only within the nervous system but is essential to all cells. Further analysis of recombinant DHPase and DRPs will reveal the biochemical nature of these molecules.

## 4. Conclusions

- (1) We have isolated cDNA clones encoding human DHPase and three human DRPs, which are homologous to non-human genes involved in nervous system development.
- (2) Mammalian *DHP*, mammalian and avian *DRP*, the gene encoding bacterial hydantoinase and *C. elegans unc-33* form a novel gene family, which we have tentatively termed the *DHP* gene family.
- (3) Human *DHP* and three human *DRP* mRNAs showed differential tissue distribution.

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**alignement des cDNA humains Ulip1-4**

hUlip-1  
hUlip-2  
hUlip-3  
hUlip-4

ATGTCCTACCAAGGCAAGAAGAACATCCGCGGATCAGAGTGACCGTCT  
ATGTCCTTATCAGGGGAAGAAAAATATTCCACGCATCACGAGCGATCGTCT  
ATGTCGTACCAGGGCAAGAAGAGCATCCCCGCATCACGAGTGACCGACT  
ATGTCCTTCCAGGGCAAGAAGCATCCCGGATCAGAGTGACCGCCT  
\*\*\*\*\*

hUlip-1  
hUlip-2  
hUlip-3  
hUlip-4

CCTTATCAAGGGAGGCAGAATCGTCAATGATGATCAGTCCTTTTATGCTG  
TCTGATCAAAGGAGGTAAATTTGTTAATGATGACCAGTCGTTCTATGCGAG  
CCTCATCAAAGGTGGACGGATCATCAACGATGACCAATCCCTTTATGCTG  
TCTGATCAGAGGTGGGAGGATCGTGAATGACGACAGTCCTTTTACGCTG  
\* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \*

hUlip-1  
hUlip-2  
hUlip-3  
hUlip-4

ATATTTACATGGAAGATGGCTTAATAAAACAAATTGGAGACAATCTGATT  
ACATATACATGGAAGATGGGTTGATCAAGCAATAGGAGAAAAATCTGATT  
ACGCTCTACCTGGAGATGGACTTATCAAAACAATAGGAGAGAACTTAATC  
ATGTGCACGTGGAAGATGGCTTGATAAAACAAATCGGAGAAACCTCATC  
\* \*

hUlip-1  
hUlip-2  
hUlip-3  
hUlip-4

GTTCTTGGAGGAGTGAAGACCATTGAAGCCAAATGGGAAGATGGTGATCC  
TGCCAGGAGGAGTGAAGACCATCGAGGCCACTCCGGATGGTGATCC  
GTTCTTGGTGGAGTGAAGACCATTGAAGCCAAACGGGCGGATGGTTATCC  
GTCTCTGGGGGCATCAAGACCATTGACGCCACGCGCTGATGGTCTTCC  
\* \*

hUlip-1  
hUlip-2  
hUlip-3  
hUlip-4

TGGAGGCATCGATGTCCATACTCACTTCCAGATGCCATATAAGGGAATGA  
CGGAGGAATTGACGTCCACACTCGTTTCCAGATGCGTGATCAGGGAATGA  
CGGAGGTATTGATGTCAACACGTACCTGCAGTAAGCCCTCCCAGGGAATGA  
TGGTGGCGCTTGACGTCCACACAAGGCTGCAGATGCTGCTCTGGGCAATGA  
\* \*

hUlip-1  
hUlip-2  
hUlip-3  
hUlip-4

CCACAGTAGATGACTTCTTCCAAGGGACAAAGCGGCCTTAGCAGGTGGC  
CGCTGCTGATGATTCTTCCAAGGAACCAAGCGGCCCTGGCTGGGGGA  
CTGCGGCTGATGACTTCTTCCAAGGGACCAAGGCGGCAGCTGGTGGCGGG  
CACCGGCTGACGACTTCTGTGTCAGGGCCACCAAGGACGCTAGCAGGAGGA  
\* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \*

hUlip-1  
hUlip-2  
hUlip-3  
hUlip-4

ACCACCATGATCATTGACCATGTGGTGCCCTGAGCCTGAGTCCAGCCTGAC  
 ACCACTATGATCATTGACCAGTTGTTCTCTGAGCCTGGGACCAAGCCTGCT  
 ACCACGATGATCATTGACCATGTTGTTTCTGACCTGGGTCCAGCCTACT  
 ACCACCATGATCTTGACCACCGTCTTCCCAGCACGGGTGTGAGCCTGCT  
 \*\*\*\*\*

hUlip-1  
hUlip-2  
hUlip-3  
hUlip-4

TGAGGCCTATGAGAAATGGAGAGAGTGGGCTGATGGGAAGATTGCTGTG  
 CGCTGCCCTTTGACCACTGGAGGGAATGGGCCGACAGCAAGTCTCTGTGTG  
 GACCTCTTTGAGAACTGGCACGAAGCAGCTGACACCAAATCCTGCTGTG  
 GGCGGCCCTACGAGCGCTGGCGGGAGCGGGCGGACAGCGCGGCTGCTGCC  
 \* \* \* \* \*

hUlip-1  
hUlip-2  
hUlip-3  
hUlip-4

ACTATGCCCTGCATGTGGACATCACCCACTGGAATGACAGCGTCAAGCAG  
ACTACTCTCTGCATGTGGACATCAGCGAGTGGCATAAGGGCATCCAGGAG  
ATTACTCCCTCCAGTGGACATCACAACTGCTACGATGGCGTTCGGGAG  
ACTACTCCCTGCACGTGGACATCACCCATGGCATGAGAGCATCAAGGAG  
\* \* \* \* \*

```
hUlip-1
hUlip-2
hUlip-3
hUlip-4
```

GAAGTGCAGAACCTCATCAAGGACAAAGGGGTAACTCCTTCATGGTTTA  
GAGATGGAAGCGCTTGTGAAGGATCACGGGGTAAATTCCTTCCTCGTGA  
GAGCTGGAGGTGCTGGTGCAGGACAAAGCGCTCAATTCCTCCAGTCTA  
GAGCTGGAGGCCCTGGTCAAGGAGAGGGGTGTAAGCTCCTTCCTGCTCT  
\* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \*





hUlip-1  
hUlip-2  
hUlip-3  
hUlip-4

TATGGCTTATAAGGATTTGTATCAAGTATCTAACACAGAGCTCTATGAGA  
CATGGCTTTCAAGATCGCTTCCAGCTAACGGATTGCCAGATTTATGAAG  
CATGGCTTATAAGGATGTCTACCAAATGTCCGACAGCCAGCTCTATGAAG  
CATGGCATACAAGGACCGGTGCCAGTGCAGCGACAGCCAGATGTACGAGA  
\*\*\*\*\*

hUlip-1  
hUlip-2  
hUlip-3  
hUlip-4

TCTTCACCTGCCTGGGAGAGCTGGGGGCCATTGCTCAAGTTCATGCTGAG  
TACTGAGTGTGATCCGGGATATTGGCGCCATAGCCCAAGTCCACGCAGAA  
CCTTTACCTTCTTAAGGGCCTGGGAGCTGTGATCTTGGTCCATGCAGAA  
TCTTCAGCATCATCCGGGACCTGGGGGCCCTTGGCCCAGGTGCACGCTGAG  
\*\*\*\*\*

hUlip-1  
hUlip-2  
hUlip-3  
hUlip-4

AATGGGGATATCATTGCCAGGAGCAAACCCGCATGTTGGAATGGGGAT  
AATGGCGACATCATTGCAGAGGAGCAGCAGAGGATCCTGGATCTGGGCAT  
AATGGAGATTTGATAGCTCAGGAACAAAGCGGATCCTGGAGATGGGCAT  
AACGGGGACATCGTGGAGGAGGAGCAGAAGCGGTTGCTGGAGCTCGGCAT  
\*\*\*\*\*

hUlip-1  
hUlip-2  
hUlip-3  
hUlip-4

AACTGGCCCAGAAGGCCATGTACTGAGCAGGCCAGAAGAGCTGGAAGCTG  
CACGGGCCCCGAGGGACATGTGCTGAGCCGACCTGAGGAGGTGAGGCGCG  
CACGGGTCCCAGGGGCCATGCCCTGAGCAGACCTGAAGAGCTGGAGGCGG  
CACTGGCCCCGAGGGCCACGTGCTCAGCCACCCGAGGAGGTGGAGGCTG  
\*\*\*\*\*

hUlip-1  
hUlip-2  
hUlip-3  
hUlip-4

AGGCTGTGTTCCGTGCCATCACCATTGCCAGCCAAACCAATTGCCCTCTC  
AAGCCGTGAATCGTGCCATCACCATCGCCAAACAGACCAACTGCCCGCTG  
AGGCGGTGTTCCGGGCCATCACCATTGCGGGCCGGATCAACTGCCCTGTG  
AGGCGGTGTACCGAGCTGTCCACATCGCCAAAGCAGGCAAACTGCCCGCTG  
\*\*\*\*\*

hUlip-1  
hUlip-2  
hUlip-3  
hUlip-4

TACGTACAAAGGTCATGAGCAAGAGTGCAGCTGACCTCATCTCACAAAGC  
TATATACCAAGGTGATGAGCAAAAGCTCTGCTGAGGTGATCGCCAGGC  
TACATACCAAGGTCATGAGCAAGAGTGCAGCCGACATCATCGCTCTGGC  
TACGTACCAAGGTGATGAGCAAGGGGCGCGCCGACGCCATCGCTCAGGC  
\*\*\*\*\*

hUlip-1  
hUlip-2  
hUlip-3  
hUlip-4

CAGGAAAAAAGGAAATGTAGTCTTTGGTGAGCCCATCACTGCCAGCCTCG  
ACCGAAGAAGGGAAGTGTGGTGTATGGCGAGCCCATCACTGCCAGCTTGG  
CAGGAAGAAGGGCCCTAGTTTTTGGAGAGCCCATTGCCCGCAGCCTGG  
CAAGCCAGAGGGGTGGTGTGTTTGGGAGCCCATCACCGCCAGCCTGG  
\*\*\*\*\*

hUlip-1  
hUlip-2  
hUlip-3  
hUlip-4

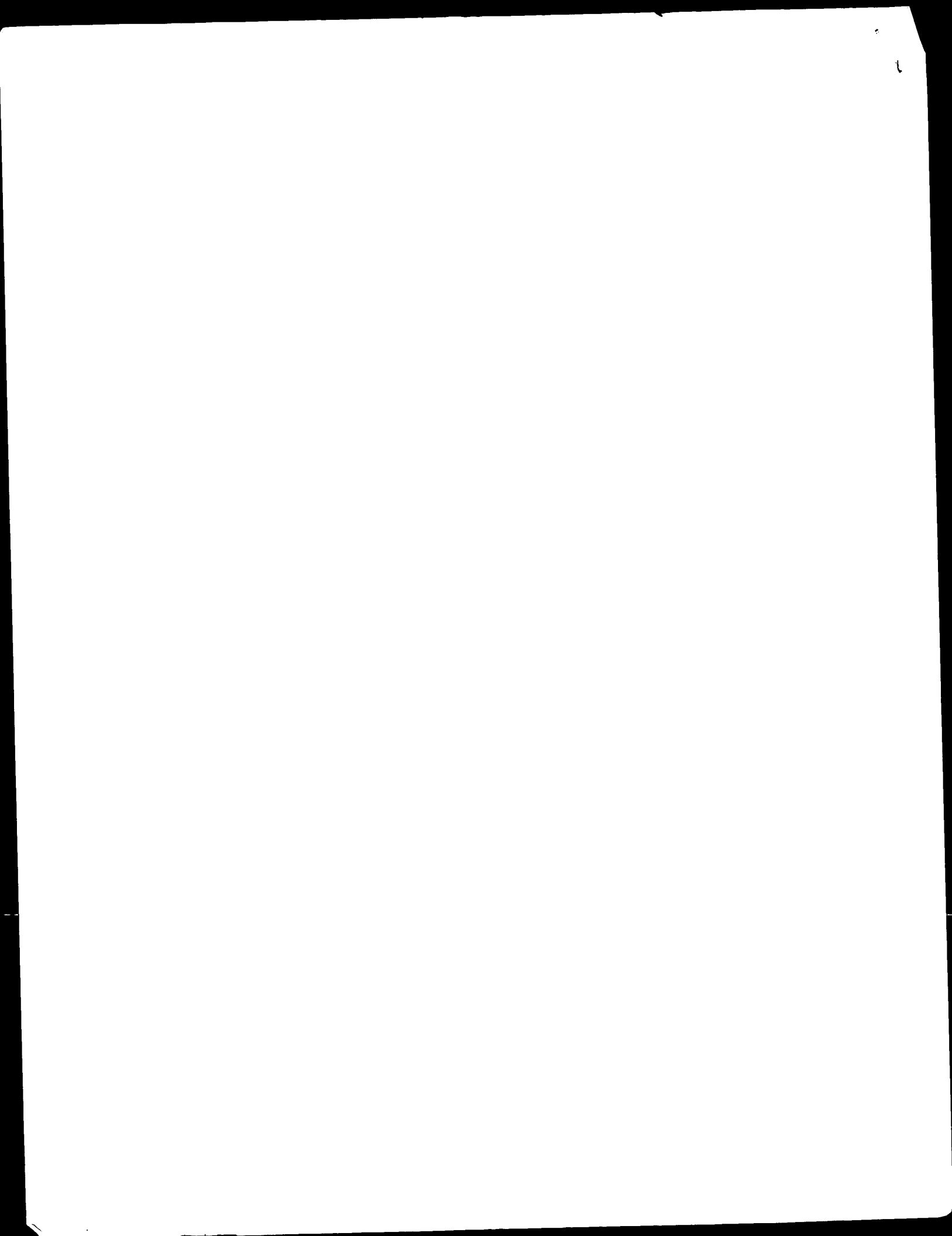
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GCACCGACGGTTACACTACTGGAGCAAGAAGTGGGCCAAGGCGCAGCC  
\*\*\*\*\*

hUlip-1  
hUlip-2  
hUlip-3  
hUlip-4

TTTGTGACATCCCCACCCCTGAGCCCTGACCCAACTACTCCGGACTACAT  
TTTGTACCTCCCCACCCCTGAGCCCTGATCCAACCACTCCAGACTTTCT  
TTCGTGACTTCCCCTCCCCTGAGCCCGACCCCTACCACGCCCGACTACTT  
TTCGTACATCAACCCCTGTCAACCCAGACCCACCCAGGCAGACCACCT  
\*\*\*\*\*

hUlip-1  
hUlip-2  
hUlip-3  
hUlip-4

CAACTCCTTGCTGGCCAGCGGGGATCTGCAGCTATCTGGGAGTGCCCACT  
CAACTCCTTGCTGTCTGTGGAGACCTCCAGGTACCGGGCAGTGCCCAT  
GACCTCCCTACTGGCCTGTGGGGACTTGCAAGGTACAGGCAGCGGCCACT  
CACCTGCTTGCTGTCCAGCGGGGACCTCCAGGTGACAGGCAGCGGCCACT  
\*\*\*\*\*



hUlip-1 GCACCTTCAGCACTGCCCAGAAAGCAATTGGGAAGGACAACCTTCACAGCC  
hUlip-2 GCACGTTTAACTGCCCAGAAAGGCTGTAGGAAAGGACAACCTTCACCCTG  
hUlip-3 GTCCCTACAGCACTGCCCAGAAAGCGGTGGGCAAGGACAACCTTACCCTG  
hUlip-4 GCACCTTCACCACTGCCCAGAAAGGCTGTGGGCAAGGACAACCTTCGCGCTG  
\* \* \* \* \*

hUlip-1 ATTCTGAGGGCACCAATGGTGTGGAGGAGCGGATGTCTGTCTATCTGGGA  
hUlip-2 ATTCCGGAGGGCACCAATGGCACTGAGGAGCGGATGTCCGTCTATCTGGGA  
hUlip-3 ATCCCCGAGGGTGTCAACGGGATAGAGGAGCGGATGACCGTCTCTGGGA  
hUlip-4 ATCCCCGAGGGCACCAACGGCATTGAGGAGCGCATGTCTGATGTCTGGGA  
\* \* \* \* \*

hUlip-1 CAAGGCTGTGGCCACAGGGAATGGACGAAAACCAAGTTTCGTGGCTGTGA  
hUlip-2 CAAGGCTGTGGTCACTGGGAAGATGGATGAGAACCAGTTTGTGGCTGTGA  
hUlip-3 CAAGGCGGTGGCTACTGGCAAAATGGATGAGAACCAGTTTGTCTGTGTCA  
hUlip-4 GAAATGTGTGGCCTCTGGGAAGATGGACGAGAATGAGTTTCGTCTGGCTGA  
\* \* \* \* \*

hUlip-1 CAAGCACAAACGCTGCCAAGATCTTCAACCTGTATCCCCGCAAGGGAAGA  
hUlip-2 CCAGCACCAATGCAGCCAAAGTCTTCAACCTTTACCCCCGAAAGGCCGC  
hUlip-3 CCAGCACCAATGCAGCCAAAGATCTTTAACCTGTACCCAAGGAAAGGGCGG  
hUlip-4 CCAGTACAAATGCTGCCAAATCTTCAATTTTACCCAAGGAAGGGGCGA  
\* \* \* \* \*

hUlip-1 ATATCTGTGGGTTCTGACAGCGACCTCGTCATCTGGGATCCAGATGTGT  
hUlip-2 ATTGCTGTGGGATCCGATGCCGACCTGGTCATCTGGGACCCCGACAGCGT  
hUlip-3 ATTGCCGTGGGCTCGGATGCCGACGTGGTCATCTGGGACCCCGACAAGTT  
hUlip-4 GTGGCTGTGGGCTCTGACGCTGACCTGGTCATATGGAACCCCAAGGCCAC  
\* \* \* \* \*

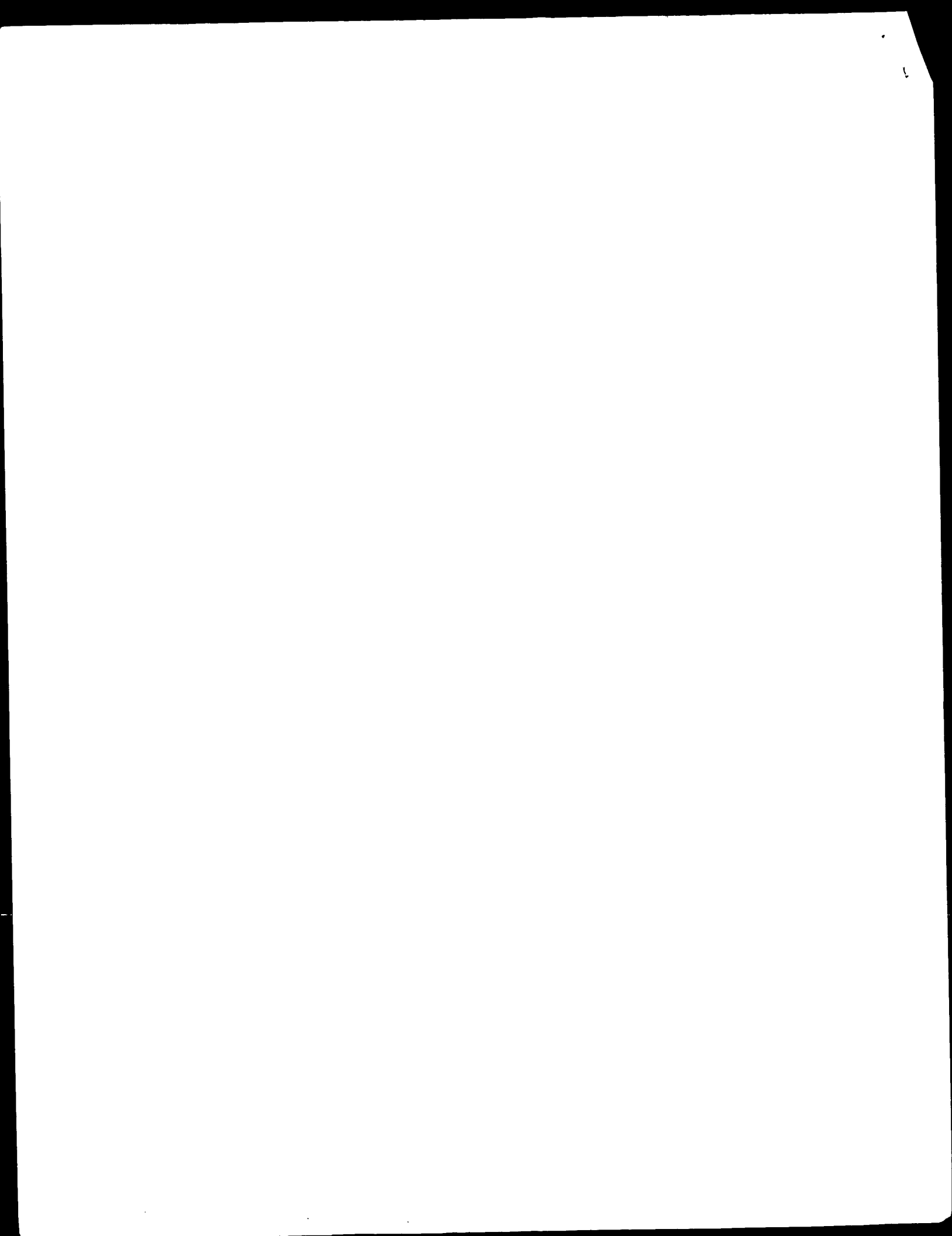
hUlip-1 GAAGATCGTCTCTGCCAAGAACCACCAAGTCTGCGGCAGAGTACAACATCT  
hUlip-2 TAAAACCATCTCTGCCAAGACACACAACAGCTCTCTCGAGTACAACATCT  
hUlip-3 GAAGACCATACAGCCAAAAGTCAAGTCCGCGGTGGAGTACAACATCT  
hUlip-4 CAAGATCATCTCTGCCAAGACCCACAATCTGAACGTGGAGTACAACATCT  
\* \* \* \* \*

hUlip-1 TTGAAGGGATGGAGCTGCGCGGGGCTCCTCTGGTTGTCTATCTGCCAGGGC  
hUlip-2 TTGAAGGCATGGAGTGCCGCGGCTCCCCACTGGTGGTCATCAGCCAGGGG  
hUlip-3 TCGAGGGTATGGAGTGCCACGGCTCCCCACTAGTGGTCATCAGCCAGGGC  
hUlip-4 TCGAGGGAGTGGAGTGCCGGGGAGCGCCTGCCGTGGTCATAAGTCAGGGC  
\* \* \* \* \*

hUlip-1 AAGATCATGCTGGAAGATGGCAACCTGCACGTGACCCAGGGGCTGGCCG  
hUlip-2 AAGATTGTCCTGGAGGACGGCACCTGCATGTACCGAAGGCTCTGGACG  
hUlip-3 AAGATCGTCTTTGAAGACGGAAACATCAACGTCAACAAGGGCATGGGCGG  
hUlip-4 CGAGTGGCGCTGGAGGACGGGAAGATGTTTGTACCCCGGGGGCGGGCCG  
\* \* \* \* \*

hUlip-1 CTTACATACCCTGCAGCCCGTTCTCCGACTATGTCTACAAGCGCATTAAAG  
hUlip-2 CTACATTCCCCGGAAGCCCTTCCCTGATTTTGTTTACAAGCGTATCAAGG  
hUlip-3 CTTCAATTCCGCGGAAGGCGTTCCCGAGCACCTGTACCAGCGCGTCAAAA  
hUlip-4 CTTCTGTCCTCGGAAACATTTCCCGACTTTGTCTACAAGAGGATCAAAG  
\* \* \* \* \*

hUlip-1 CACGGAGGAAGATGGCAGACCTGCATGCCGTCCCAAGGGGCATGTACGAT  
hUlip-2 CAAGGAGCAGGCTGGCTGAGCTGAGAGGGGTTCCCTGGGCTGTATGAC  
hUlip-3 TCAGGAATAAGGTTTTTGGATTGCAAGGGGTTTCCAGGGGCATGTATGAC  
hUlip-4 CTCGCAACAGGCTGGCGGAGATCCACGGTGTGCCCGTGGGCTGTATGAC  
\* \* \* \* \*



hUlip-1 GGGCCTGTGTTGACCTGACCACCACCCCAAAGGTGGGACCCCGCAGG  
hUlip-2 GGACCTGTGTGTGAAGTGTCTGTGACGCCCAAGACAGTCACTCCAGCCTC  
hUlip-3 GGTCTGTGTACGAGGTACCAGCTACACCCAAATATGCAACTCCCCTCC  
hUlip-4 GGGCCCGTCCACGAGGTGATGGTGCCTGCCAAGCCAGGGAGTGGCGCTCC  
\* \* \* \* \*

hUlip-1 CTCTGCTCGGGGCTCTCCTACT---CGGCCGAACCCACCTGTGAGGAATC  
hUlip-2 CTCGGCCCAAGACGTCTCCTGCCAAGCAGCAGGCCCCACCTGTCCGGAACC  
hUlip-3 TTCAGCCAAATCTTCGCCTTCTAAACACCAGCCCCCACCATCAGAAACC  
hUlip-4 GGCCCGCGCGTCTTCCCCAGGCAAGATCTCCGTGCCTCCTGTGCGCAACC  
\* \* \* \* \*

hUlip-1 TTCATCAGTCGGGATTTAGCCTGTCAGGCACCCAAAGTGGATGAGGGGGTT  
hUlip-2 TGCACCACTCTGGATTCTAGTTTGTCTGGTGCTCAGATTGATGACAACATT  
hUlip-3 TCCACCACTCCAACCTCAGCTTATCAGGTGCCAGATAGATGACAACAAT  
hUlip-4 TACATCAGTCGGGGTTACGCCTATCTGGGTCTCAGGCTGATGACCACATC  
\* \* \* \* \*

hUlip-1 C---GCTCAGCCAGCAAGCGCATCGTGGCCCCCCCCAGGCGGCCGTTCTAA  
hUlip-2 CCCC GCCG CACCACCCAGCGTATCGTGGCGCCCCCGGTGGCCGTGCCAA  
hUlip-3 CCCAGGCGCACC GCCCACC GCATCGTGGCGCCCCCTGGTGGCCGCTCCAA  
hUlip-4 GCCCGACGCACAGCACAGAAGATCATGGCACCACCTGGCGGCCGCTCCAA  
\* \* \* \* \*

hUlip-1 TATCACATCTCTGAGTTAA  
hUlip-2 CATCACCAGCCTGGGCTAG  
hUlip-3 CATCACCAGCCTCGGTTGA  
hUlip-4 CATCACCTCTCTCCTAG  
\*\*\*\*\*

Sequences (U1:U2) Aligned. Score: 68.7682  
Sequences (U1:U3) Aligned. Score: 67.0169  
Sequences (U1:U4) Aligned. Score: 64.6235  
Sequences (U2:U3) Aligned. Score: 70.1571  
Sequences (U2:U4) Aligned. Score: 69.0518  
Sequences (U3:4) Aligned. Score: 65.2705

